# The regulatory network controlling DNA damage responses in *Saccharomyces cerevisiae*

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# ABSTRACT

DNA is subject to attack by DNA damaging agents from both environmental and endogenous sources. In response to DNA damage, living organisms enhance expression of many related genes to facilitate DNA repair and survival. The SOS response is a well-understood prokaryotic regulatory cascade that controls the expression of more than 30 genes in response to DNA damage. However, in eukaryotic organisms from simple budding yeast to human, such a regulatory network has not been reported.

Previous research in our laboratory found that among DNA repair mutants of *Saccharomyces cerevisiae*, only *rad6* and *rad18* defective in the post-replication repair pathway significantly affected DNA damage induction of several genes examined. Rad6 and Rad18 form a ubiquitin conjugation-ligase complex and are required for the cellular tolerance to damaged DNA. Since the Rad6-Rad18 complex binds to single-stranded DNA, it may act as a DNA damage sensor required for the activation of DNA damage-induced transcription. We performed microarray analysis and found that the induction of up to 379 genes, including those involved in DNA repair, control of replication and transcription, regulation of the cell cycle and cell metabolism, are compromised in the *rad6* and *rad18* mutants. Although Rad6/Rad18 monoubiquitinates proliferating cell nuclear antigen (PCNA) following DNA damage to initiate a damage tolerance response, PCNA ubiquitination is not required for DNA damage induction. In budding yeast, cell-cycle checkpoints are involved in the control of DNA damage induction of gene expression through phosphorylation of a protein kinase Rad53 by two pathways represented by Rad24 and Sgs1. The Rad6-Rad18 complex appears to function in the Rad24 pathway and parallel to Sgs1. We further demonstrated that the Rad17 subunit of

the 9-1-1 complex is subject to Rad6/Rad18- and DNA damage-dependent mono-ubiquitination and that the Rad17-Lys197 residue with flanking sequences homologous to Lys164 of PCNA is absolutely required for the DNA damage induction by Rad6-Rad18. Hence, by ubiquitinating two DNA clamps, PCNA and 9-1-1, the Rad6-Rad18 complex plays a central role in the cellular response to DNA damage by coordinating translesion synthesis, error-free bypass, homologous recombination, as well as transcriptional regulation, reminiscent of roles of RecA in *E. coli* cells.

Several individual genes have also been examined in this study to elucidate the regulatory mechanisms acting on specific DNA damage-inducible genes. In the microarray analysis, *DDI2* and *DDI3,* two identical genes located in duplicated chromosomal regions, were identified due to the highest induction ratio (122-fold) after MMS treatment. Interestingly, *DDI2*/*DDI3* can only be highly induced by $SN_2$-type alkylating agents. Promoter deletion analysis mapped the putative upstream acting sequence ($UAS_{DDI2}$) responsible for 40% of basal expression and 90% of induced expression by MMS.

The *CRT10* gene was identified through screening of the yeast deletion library for hydroxyurea (HU) resistance. *CRT10* encodes a putative 957 amino acid, 110 kDa protein with a leucine repeat and a WD40 repeat near the N-terminus. Deletion of *CRT10* resulted in an enhanced resistance to HU reminiscent of the inactivation of two other ribonucleotide reductase (Rnr) suppressors, *CRT1* and *SML1*, which regulate Rnr activity at transcriptional and translational levels, respectively. Epistasis analysis indicates that *CRT10* belongs to the *CRT1* pathway but not the *SML1* pathway. Indeed, deletion of *CRT10* enhanced the survival of the *mec1* null mutant and increased basal level and DNA

damage-induced expression of *RNR2* and *RNR3*, suggesting that Crt10 regulates *RNR* genes at the transcriptional level. Furthermore, the *dun1* mutation is epistatic to *crt10* with respect to both HU sensitivity and *RNR* gene expression. Interestingly, the expression of *CRT10* itself is induced by DNA damaging agents and this induction requires *DUN1*, suggesting that *CRT10* plays a role in cellular response to DNA damage and replication blocks. The *CRT10* function appears to be achieved by positive regulation of the *CRT1* transcript level, indicating that *CRT10* is a component of the regulatory circuit.

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*To my family*

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| | |
|---|---|
| 3-AT | 3-aminotriazole |
| 4-NQO | 4-Nitroquinoline-*N*-oxide |
| 6-4 PP | pyrimidine-pyrimidone photoproduct |
| 9-1-1 | Rad9-Rad1-Hus1 |
| aa | amino acid |
| Amp | ampicillin |
| AP | apurinic/apyrimidinic |
| AT | ataxia telangiectasia |
| ATF-2 | activating transcription factor 2 |
| ATM | Ataxia-telangiectasia mutated |
| ATR | ATM and Rad3-realted |
| BCNU | 1,3-*N*,*N*'-bis(2-chloroethyl)-*N*-nitrosourea |
| BER | base excision repair |
| BIR | break-induced replication |
| β-gal | β-galactosidase |
| bZIP | basic-leucine zipper |
| CBP | cAMP response element-binding protein |
| ChIP | chromatin immunoprecipitation |
| co-IP | co-immunoprecipitation |
| CPD | cyclobutane pyrimidine dimer |
| CRE | cAMP responsive element |
| Ct | cross threshold |
| dam | DNA adenine methylase |
| $ddH_2O$ | double distilled water |
| dNDP | deoxynucleoside diphosphate |
| DMS | dimethyl sulfonate |
| DMSO | dimethyl sulfoxide |
| DSB | double-stranded DNA break |
| dsDNA | double stranded DNA |
| DTT | dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| EMS | ethyl methanesulfonate |
| EPR | enzymatic photoreactivation |
| EtBr | ethidium bromide |
| GGR | global genome repair |

| | |
|---|---|
| HD | Histidine-Aspartic acid |
| HR | homologous recombination |
| HU | hydroxyurea |
| Ile | isoleucine |
| IP | immunoprecipitation |
| JNK | JUN N-terminal kinase |
| K | lysine |
| Kb | kilobase pair |
| kDa | kilodalton |
| LB | lysogeny broth |
| Leu | leucine |
| LFS | Li-fraumeni syndrome |
| Lys | lysine |
| MAF | musculoaponeurotic fibrosarcoma oncogene family |
| *MAT* | mating type locus |
| Met | methionine |
| MMR | mismatch repair |
| MMS | methyl methanesulfonate |
| MNNG | *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine |
| NDP | nucleoside diphosphate |
| NEM | N-ethylmaleimide |
| NES | nuclear export sequence |
| NER | nucleotide excision repair |
| NHEJ | non-homologous end joining |
| nt | nucleotide |
| OD | optical density |
| ORF | open reading frame |
| PBS | phosphate buffer saline |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PDGF | platelet derived growth factor |
| PI-3K | phosphoinositide-3 kinase |
| PCNA | proliferating cell nuclear antigen |
| PRR | post-replication repair |
| R | arginine |
| RFC | replication factor C |
| RNR | ribonucleotide reductase |
| ROS | reactive oxygen species |
| RPA | replication protein A |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |

| | |
|---|---|
| SD medium | synthetic dextrose medium |
| SDS | sodium dodecyl sulfate |
| SDSA | sythesis-dependent strand annealing |
| *S. pombe* | *Saccharomyces pombe* |
| SSA | single-strand annealing |
| SSB | single-stranded DNA break |
| ssDNA | single stranded DNA |
| SUMO | small ubiquitin-related modifier |
| t-BuOOH | tert-butyl hydroperoxide |
| TCA | trichloroacetic acid |
| TCR | transcription-couple repair |
| TLS | translesion DNA synthesis |
| Trp | tryptophan |
| UAS | upstream activating sequence |
| Ub | ubiquitin |
| UES | upstream essential sequence |
| Ura | uracil |
| URS | upstream repressing sequence |
| UV | ultraviolet radiation |
| WD | Tryptophan-Aspartic acid |
| Wt | wild type |
| YPD | yeast extract-peptone-dextrose |

# CHAPTER ONE

# INTRODUCTION

## 1.1. DNA damage and repair pathways

DNA is the carrier of genetic information in most organisms. In general, alterations in the chemistry or sequence of DNA are appropriately considered to be DNA damage. Any damage to the molecular structure of DNA has the potential to cause genomic instability, mutagenesis or even cell death. Unfortunately, DNA damage is unavoidable: DNA is continually exposed to insults resulting from exogenous and endogenous DNA damaging agents as well as by challenges posed by DNA replication. Therefore, it is not surprising that cells have evolved a multitude of mechanisms to remove the damaged DNA and restore the normal nucleotide sequence and DNA structure.

### 1.1.1. DNA damage

DNA damage is normally divided into two major classes, namely endogenous DNA damage and environmental DNA damage. Endogenous DNA damage includes the DNA damage caused by reactive species generated during cellular metabolism, hydrolytic damage and DNA replication errors. Environmental DNA damage is referred to that caused by physical and chemical agents generated outside cells.

*In vivo*, DNA reacts with oxygen and water, leading to many spontaneous DNA lesions. Cytosine can spontaneously deaminate to uracil, thus resulting in G·C to A·T mutations. Adenine and guanine can also deaminate but at rates much lower than

cytosine deamination (Friedberg *et al*., 2006). Oxidation of DNA by reactive oxygen species (ROS) which are generated in the cells by normal aerobic metabolism is one of the major types of endogenous DNA damage. Oxidative DNA damage is the main cause for cancer and neurological diseases in human, and it is closely related to the normal process of ageing. The ROS abstracts hydrogen atoms from the deoxyribose sugar or is added to double bonds of DNA bases, therefore causing oxidised base residues, base losses and single-strand breaks (Bjelland and Seeberg, 2003). Insertion of an incorrect base during DNA replication is another source of DNA alteration, resulting in a nucleotide mismatch. Although many DNA polymerases can synthesize DNA with great accuracy, the intrinsic error frequency for DNA replication polymerases is still between $10^{-6}$ and $10^{-7}$ (Kunkel and Bebenek, 2000). In addition, under conditions such as an imbalanced dNTP pool (Kunkel et al., 1982) or the absence of certain accessory proteins (Kunkel et al., 1979), the error rate is increased by 100-1000 fold.

Many environmental DNA damaging agents induce a variety of DNA lesions. Two major physical DNA damaging agents are ionizing radiation and UV irradiation. Modified DNA bases and strand breaks constitute the two major classes of DNA damage by ionizing radiation. Ionizing radiation can directly break DNA strands through absorption of the radiation energy, and most of the lethal effects can be attributed to these lesions (Ward, 1988). Furthermore, radiation can lead to the formation of molecules, such as ·OH radicals, by acting with water or other surrounding molecules, and these species cause several kinds of DNA lesions (Friedberg et al., 2006). As for UV irradiation, it frequently causes cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone photoproduct (6-4 PP). DNA cross-links, DNA-protein cross-links and

strand breaks are also observed after UV irradiation, but much less prominently (Friedberg et al., 2006).

There are many chemical agents that can damage DNA. For example, alkylating agents such as methyl methanesulfonate (MMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) are electrophic compounds with affinity for nucleophilic centers in DNA (Friedberg et al., 2006). Numerous potential reaction sites for alkylation have been identified in all four bases with different affinity: in adenine, N1, N3, N7 and N6; in guanine, N1, N3, N7, N2 and $O^6$; in cytosine, N3, N4 and $O^2$; in thymine, N3, $O^2$ and $O^4$ (Friedberg et al., 2006). The alkylating adducts can block DNA synthesis or form mismatches leading to transition mutations (Lindahl et al., 1988; Loveless, 1969; Preston et al., 1986), therefore alkylating agents (e.g. MMS) are frequently used in DNA repair studies as DNA damaging agents and will be often referred to in this thesis. Cross-linking agents such as cisplatin and nitrogen mustard can form intrastrand cross-links or interstrand cross-links, thus completely blocking DNA replication and transcription (Eastman, 1987; Kohn et al., 1966). Moreover, some chemicals like the anti-tumor antibiotic bleomycin and enediyne induce hydrogen abstraction from DNA, leading to a mixture of strand breaks and abasic sites (Povirk, 1996).

### 1.1.2. DNA repair pathways

DNA repair is the cellular response to DNA damage that restores the altered nucleotide sequence and DNA structure to its native state (Friedberg et al., 2006). The major pathways include base damage reversal, base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), recombination repair and

postreplication repair (PRR). These DNA repair pathways are highly conserved from bacteria to mammals. In the vast majority of cases, proteins that carry out these repair pathways are conserved in structure and function in eukaryotes, as well as in some cases in bacteria. Defects in these pathways would lead to an accumulation of mutations in genomic DNA so as to increase the frequencies of mutagenesis and cell death.

#### 1.1.2.1. Base damage reversal

Base damage reversal is usually carried out by a single polypeptide enzyme, which catalyzes a single-step reaction to repair the damaged DNA (particularly damage to the nitrogenous bases) (Friedberg et al., 2006). A good example of this pathway is the enzymatic photoreactivation (EPR) of base damage caused by UV irradiation. As mentioned before, adjacent pyrimidines would be covalently linked to form CPD or (6-4) PP when DNA is exposed to UV irradiation. These photoproducts can interfere with DNA replication and transcription, thus threaten the viability and normal functions of cells (Moore and Strauss, 1979; Setlow et al., 1963; Villani et al., 1978). EPR can specifically reverse the pyrimidine dimers to native pyrimidine monomers. This process is catalyzed by photolyases which require light in a particular range of wavelengths. In late 1960s, a yeast mutant defective in EPR established the existence of the *PHR1* gene (Resnick, 1969). The *PHR1* gene encodes a 66kDa pyrimidine dimer DNA photolyase which is able to catalyze EPR (Sancar, 1985; Yasui and Langeveld, 1985). Later research showed that the transcription of *PHR1* is induced by various DNA damaging agents (Sebastian et al., 1990).

In addition to EPR reversing base damage caused by UV irradiation, some alkylation base damage can also be directly reversed by enzyme-catalyzed processes. For

example, *$O^6$-methylguanine*-DNA alkyltransferase ($O^6$-MGT) can transfer methyl groups from alkylation damage products *$O^6$-methylguanine* to protect cells against DNA alkylation damage (Samson and Cairns, 1977; Samson et al., 1986; Sassanfar and Samson, 1990). The predicted amino acid sequences of bacterial, yeast and human *$O^6$*-MGT genes are highly conserved (Xiao et al., 1991).

#### 1.1.2.2. Base excision repair (BER)

BER is a process to remove damaged bases from DNA and replace them with pristine sequences. BER is the major repair pathway against DNA damage such as deaminated, oxidized, alkylated bases and abasic sites which are normally generated by reactive oxygen species or alkylating agents (Memisoglu and Samson, 2000), and it is probably one of the most highly conserved and most frequently used DNA repair modes in nature.

BER is a multi-step process in which the first step is recognition and removal of the damaged base. DNA glycosylase removes the damaged base through cleavage of the N-glycosylic bond connecting the base and the sugar-phosphate backbone. The second step is incision at the resulting abasic (or AP) site. AP endonuclease (APE) recognizes an AP site and cleaves the DNA phosphodiester backbone on the 5' side of the AP site, leaving a 3'-hydroxyl terminus and a 5'-deoxyribose phosphate (dRp) terminus flanking the nucleotide gap. Replacement of the missing nucleotide(s) is the third step. This repair step can proceed via two sub-pathways that utilize different subsets of enzymes: one is short-patch BER that involves replacement of 1 nt; the other is long-patch BER that involves gap-filling of 2–6 nt. In short-patch BER, polymerase β removes the 5'-dRp moiety by its intrinsic dRp lyase activity and adds one nucleotide into the repair gap. In

long-patch BER, polymerase δ/ε extends the repair patch and displaces several nucleotides to create a 5'-flap that is cleaved by the flap endonuclease FEN1 to create a ligatable nick. The last step is sealing of the final nick (Chan et al., 2006; Fortini et al., 2003; Memisoglu and Samson, 2000; Wilson and McNeill, 2006).

#### 1.1.2.3. Mismatch repair (MMR)

MMR targets base-base mismatches, and it is essential to all organisms since it maintains the stability of the genome during repeated duplication. Defects in the mismatch repair pathway elevate spontaneous mutability 50 to 1000 fold (Kolodner, 1996; Schofield and Hsieh, 2003).

MutS, MutL, MutH, and UvrD, which are essential components of the MMR pathway, were identified in *E. coli* through the genetic studies of mutants that showed elevated mutation levels (Cox et al., 1972; Wagner and Meselson, 1976). MutS initiates repair by binding to the mismatch base, then forms a heteroduplex complex with MutL to activate a methyl-directed endonuclease system, which incises at the hemimethylated dam site of unmethylated strand with MutH and UvrD (Au et al., 1992; Iyer et al., 2006; Modrich and Lahue, 1996). MutS and MutL homologs have been identified in all eukaryotic organisms, including yeast, mouse and human. However, no MutH and UvrD homologs have been found in eukaryotic genomes (Jun et al., 2006). In eukaryotic cells, the MMR pathway requires not only MutS homologs (MSH2-6) and MutL homologs (MLH1-3, PMS1) but also proliferating cell nuclear antigen (PCNA), exonuclease I (ExoI), replication protein A (RPA), and the DNA polymerase δ (Flores-Rozas et al., 2000; Longley et al., 1997; Ramilo et al., 2002).

#### 1.1.2.4. Nucleotide excision repair (NER)

NER is a complex process to remove many helix-distorting lesions such as UV or UV-mimetic agent-induced damage and chemical adducts. NER begins with the specific recognition and verification of damage, and then removes a 24-32 base oligonucleotide from the strand containing the lesion through incision on both 5' and 3' sides. At the end, the resulting single-strand gap is filled by DNA synthesis and ligation.

In *E. coli*, UvrA, UvrB, and UvrC can locate a lesion and carry out a dual incision to remove a segment of nucleotides containing the damage. In eukaryotes, from yeast to human cells, more delicate protein complexes are used to carry out NER. For example, in budding yeast, at least seven proteins (Rad1, Rad2, Rad3, Rad4, Rad10, Rad14 and Ssl2) are required for recognition and incision of damaged DNA (Prakash et al., 1993). In human cells, XPA, RPA, the XPC-hHR23B complex, 6-9 subunits of the TFIIH complex, and two nucleases, XPG and the heterodimeric ERCC1-XPF, are required for excision of damage, and another dozen or so polypeptides are needed for the repair synthesis step (Lindahl and Wood, 1999).

Two modes of NER can be distinguished: repair of lesions over the nontranscribed strand of active genes or inactive regions of the genome is referred to global genome repair (GGR); lesions in the transcribed strands are removed by transcription-coupled repair (TCR). Studies showed that TCR removes lesions much faster than GGR does.

#### 1.1.2.5. Recombination repair

Recombination repair is the only DNA repair pathway which is able to repair double-strand breaks (DSBs). Recombination repair is classified into two categories: homologous recombination (HR) and non-homologous end joining (NHEJ).

HR uses an intact homologous region as the template to carry out DNA repair. Based on the repair mechanisms, it can be further divided into four sub-categories, namely the DSB repair model of Szostak, synthesis-dependent strand annealing (SDSA), break-induced replication (BIR), and single-strand annealing (SSA). HR requires genes in the *RAD52* epistasis group, such as *RAD50*, *RAD51*, *RAD52, RAD54, RAD55, RAD57, RAD59, MRE11*, and *XRS2*. *RAD52* is required for all forms of HR, while *RAD51*, *RAD54*, *RAD55*, *RAD57* and *RAD59* are only involved in certain forms (Aylon and Kupiec, 2004). HR is the prevalent mechanism for repairing DSBs in *S. cerevisiae*.

NHEJ directly ligates two broken DNA ends with no or minimal homology. Since no intact DNA template is involved in this process, it might be error-prone. NHEJ requires the DNA-end binding Ku complex, a DNA-dependent protein kinase (DNA-PKcs), potential DNA-end processing enzymes and the XRCC4–ligase IV complex (Ataian and Krebs, 2006; Burma et al., 2006; Daley et al., 2005). In mammalian cells, NHEJ is the predominant mechanism for quick response to DSBs, and most DSBs are repaired by this pathway rather than HR.

#### 1.1.2.6. Post-replication repair (PRR)

Studies in the early 1970s found that mammalian cells were able to complete DNA replication in the presence of persisting damage on the parental strand (Lehmann, 1972). Hence, a mechanism called post-replication repair (PRR) was suggested, which

refers to a DNA damage tolerance pathway that permits lesions bypass during replication and delayed repair. The PRR pathway encompasses two sub-pathways, namely, the error-free pathway and the error-prone pathway. The error-prone pathway employs translesion DNA synthesis (TLS) which can replace the replicative polymerase by non-essential polymerases capable of replicating over damaged regions of DNA with reduced fidelity. The error-free pathway is likely to rely on an undamaged homologous template derived from the newly made daughter strand (template switch) or from the homologous chromosome (strand exchange) for bypassing lesions (Barbour and Xiao, 2003; Broomfield et al., 2001; Laan et al., 2005).

In *E. coli*, PRR relies on some SOS response controlled gene products to act in RecA-mediated homologous recombination and TLS for lesion bypass (Friedberg et al., 2006). PRR of *S. cerevisiae* is controlled by the Rad6/Rad18 complex, and PRR is often called the *RAD6* epistatic pathway. The PRR is initiated by the binding of the Rad6/Rad18 complex to a single-strand DNA gap formed at a stalled replication fork. The Rad6/Rad18 complex is capable of mono-ubiquitinating PCNA (encoded by *POL30*) at Lys164 and results in error-prone repair which involves the TLS polymerases Polη (encode by *RAD30*) and Polζ (encoded by *REV3* and *REV7*) (Hoege et al., 2002; Stelter and Ulrich, 2003). Acting together with Rad6/Rad18, the Rad5, Mms2/Ubc13 complex is also able to poly-ubiquitinate PCNA at Lys164 to form K63-linked poly-ubiquitin chains. Poly-ubiquitination of PCNA will lead to error–free PRR pathway which could involve template switching with Rad51-independent strand exchange (Hoege et al., 2002; Pfander et al., 2005). Yeast PCNA can also be alternatively modified by the small ubiquitin-related modifier protein SUMO at K164 (major SUMO acceptor site) or K127 (minor SUMO acceptor site), and this process requires the SUMO ligase Siz1.

Sumoylation of PCNA could process a recombination-dependent error-free pathway, which would require Rad51 and would be inhibited by Srs2 (Papouli et al., 2005; Pfander et al., 2005). Almost all yeast PRR pathway genes have mammalian homologs, including *hREV1*, *hREV3, hREV7*, *hRAD30/XPV*/Polη, *hRAD30B*/Polι, *HR6A*, *HR6B*, *hRAD18*, *hMMS2* and *hUBC13*. Some of these genes are able to functionally complement the corresponding yeast null mutants, hinting the same mechanisms might apply to mammalian PRR (Broomfield et al., 2001).

## 1.2. The SOS regulatory network in *E. coli*

Under normal growth conditions, it is not necessary to maintain a high level of DNA repair activities in cells. However, after exposure to DNA-damaging agents, both prokaryotic and eukaryotic cells activate stress responses which result in specific alteration in patterns of gene expression and an active inhibition of cell division.

When *E. coli* cells are subjected to DNA damage, about 48 unlinked genes are co-ordinately induced through a complex SOS regulatory network (Courcelle et al., 2001). The increased expression of these SOS genes results in the elaboration of a set of physiological responses such as an enhanced capacity for recombination repair and excision repair, enhanced mutagenesis (due to error-prone TLS), and inhibition of cell division. These responses have been termed the SOS responses (Friedberg et al., 2006).

### 1.2.1. Current model for transcriptional control of the SOS response

The SOS regulatory network is controlled by two proteins, RecA and LexA (Cox, 2003; Janion, 2001). LexA is a transcriptional repressor which binds to SOS boxes located near or inside the operator site of the SOS-induced genes. SOS boxes are often

palindromic structures with a high degree of identity in nucleotide sequence. An ideal symmetrical consensus sequence (TACTGTATATATATACAGTA) was derived from the analysis of a pool of SOS boxes (Berg, 1988). The sequence distinction in SOS boxes allows LexA repressor to bind to operators with different strengths (Lewis et al., 1994). The binding of LexA prevents accessibility to RNA polymerase so as to inhibit the transcription initiation. LexA is likely to interact with DNA via its N-terminal domain, and the C-terminus of LexA is required for its dimerization. The dimerization of LexA is essential for its ability to repress SOS-regulated genes *in vivo* (Bertrand-Burggraf et al., 1987; Hurstel et al., 1986; Hurstel et al., 1988; Little and Hill, 1985; Schnarr et al., 1991). Meanwhile, LexA is able to undergo a slow intramolecular self-cleavage termed autodigestion, and the rate significantly increases upon interaction with RecA (Little, 1984; Little, 1991; Little, 1993).

The RecA protein of *E. coli* has at least three functions in the SOS response. It not only plays a role in the regulation of DNA damage induction, but also directly participates in TLS and homologous recombination. Following DNA damage, DNA synthesis becomes discontinuous and ssDNA is produced by failed attempts to replicate damaged DNA. In the presence of ATP, RecA binds to the ssDNA region and form the helical RecA-ssDNA nucleoprotein filaments. LexA then diffuses to deep grooves in the RecA-ssDNA filaments and interacts with them in a manner that results in autocatalytic cleavage of LexA at a scissile peptide bond located between Ala84 and GLy85. Cleavage of LexA inactivates its ability to be a repressor, thus releasing the genes from transcription repression. As cells begin to recover from the inducing treatment by various DNA repair and tolerance processes, the regions of ssDNA disappear, and thus the inducing signal is eliminated. Without the cleavage stimulated by RecA-ssDNA

filaments, the pool of LexA is boosted, which leads to repression of the transcription of the SOS regulon genes and a return to the uninduced state (Friedberg et al., 2006; Walker, 1984).

### 1.2.2. The inducing signal for SOS response

Since the SOS response can be induced by a wide variety of chemicals and some conditions, it has been a challenge to determine the nature of the signal(s) responsible for SOS induction. Many studies suggest that the DNA lesions themselves are not sufficient to induce the SOS response. The initial study took advantage of a *dnaC28*(Ts) *uvrB* double mutant (Salles and Defais, 1984). In this mutant strain, the initiation of DNA replication is temperature sensitive. When temperature is shifted to 42$^{o}$C (the non-permissive temperature), cells can still complete the existing round of replication but can not initiate a new round. UvrB is a component of the UvrABC endonuclease, and the *uvrB* mutation inactivates the NER pathway so that the UV-induced lesions will not be removed. Under permissive temperatures, the UV irradiation treatment can successfully induce an SOS response in the mutant. However, when shifting to the non-permissive temperature, no SOS response is observed in this mutant after UV irradiation, indicating that the mere presence of UV irradiation-induced lesions is not sufficient to promote an SOS response (Salles and Defais, 1984). Meanwhile, this research also suggests that the SOS response induced by UV requires an active replication fork, supporting the model that DNA replication leaves gaps where elongation stops at damage-induced lesions, and thus allows the ssDNA to activate SOS response. In addition, bleomycin, an agent known to produce DNA single-strand breaks can induce the SOS response in such a case

(Salles and Defais, 1984). This result further provides evidence that ssDNA rather than DNA lesions is required for the SOS response.

The question arises as to why the single-stranded regions normally presented on the lagging strand during normal DNA replication do not activate the SOS response. The reason is kinetic, namely, RecA might not polymerize on this DNA region, or perhaps can not displace the single-strand binding (SSB) protein from it (Sassanfar and Roberts, 1990). The hypothesis is consistent with some studies on altered RecA protein. It has been found that strains containing a *recA441* mutation activate the SOS response even without any DNA damaging treatment (Phizicky and Roberts, 1981). The RecA441 protein can bind ssDNA more tightly than the wild type RecA protein. Thus it is possible that RecA441 activates the SOS response by polymerizing on the lagging-strand gaps in normal replication (Phizicky and Roberts, 1981). Similarly, a *recA730* mutation results in constitutive SOS induction in the absence of DNA damage. The fact that RecA730 is more proficient than the wild-type RecA protein in the competition with SSB for ssDNA binding sites, also supports this model (Lavery and Kowalczykowski, 1992).

### 1.2.3. Fine tuning in the induction of the SOS response

SOS RecA/LexA regulatory system provides *E. coli* with a rapid transcriptional response to the presence of DNA damage. Furthermore, during SOS induction, the timing, the duration and the level of induction are diverse for different LexA regulated genes, suggesting a fine tuning mechanism in the SOS induction. The fine tuning is possibly determined by at least four parameters: (i) the binding affinity of LexA for the SOS box in the operator region; (ii) the number of SOS boxes in the operator region, (iii) the location of the SOS box relative to the promoter and (iv) the strength of the promoter.

After exposing *E. coli* cells to DNA damaging agents, genes with operators that bind LexA relatively weakly are the first to turn on fully. For example, *uvrA, uvrB, ruvA, ruvB, recN*, and *sulA* are induced within 5 minutes after 40 J/m$^2$ UV radiation (Courcelle et al., 2001). *uvrA* and *uvrB* encode proteins involved in NER; *recN, ruvA* and *ruvB* encode proteins used for recombination repair; the protein product of the *sulA* gene can temporarily arrest cell division so that it gives the bacteria time to complete the repair of damaged DNA. After the activation of loosely controlled SOS genes, if the damage can not be fully repaired by NER and homologous recombination, genes with operators that are tightly controlled by LexA will be turned on. For example, research shows that the full induction of *umuC* and *umuD* is not observed until 20 minutes after UV irradiation (Courcelle et al., 2001). Similar to LexA, the protein encoded by *umuD* also has a latent ability to autodigest, and the autodigestion is strongly stimulated by the interaction between the RecA/ssDNA nucleoprotein filament and UmuD (Nohmi et al., 1988). UmuC and a post-translationally processed form of UmuD (UmuD') serve as a mutagenic lesion-bypass DNA polymerase. This last response allows the survival of *E. coli* after severe DNA damage, but at the expense of introducing errors into the genome.

In the SOS regulatory network, the transcription of the key regulatory gene *recA* is also regulated by LexA, thus forming a delicately controlling circuit. *recA* has one SOS box which is located between the -35 and -10 region of the promoter, and LexA can bind to this region to prevent the initiation of transcription (Schnarr et al., 1991). Relative binding affinity experiments have shown that LexA binds to the *recA* operator more strongly than the operators of many other SOS regulon genes such as *uvrA*, *uvrB* and *uvrD* (Schnarr et al., 1991). Accordingly, it allows an intermediate inducible state between an uninduced state and a fully induced state. A low amount of inducing signal

thus can lead to the activation of some of the SOS functions, such as *uvr*$^+$ dependent NER, without substantial amplification of the RecA protein. When the inducing signal is not limiting, the expression of *recA* will be induced and result in a full induction of SOS genes. Furthermore, the strong repression of *lexA* to *recA* ensures a fast return to the uninduced state once the level of the inducing signal begins to decrease (Walker, 1984).

In addition to two central regulators RecA and LexA, recent studies have shown that some other proteins are also involved in the subtleties of SOS induction. All these proteins affect SOS regulation by modulating RecA-ssDNA stability. RecX can block the assembly of RecA-ssDNA filament while not affecting the disassembly through capping the assembly ends (Drees et al., 2004). As a result, it strongly inhibits RecA-mediated DNA strand exchange, ATPase, and coprotease activities. The *recX* gene is located 76bp downstream of *recA*, and these two genes belong to the same operon. *recX* is co-transcribed with *recA*, however *recX* transcription is down regulated with respect to *recA* by an intrinsic transcription terminator that is located between the *recA* and *recX* coding sequences. Despite the presence of this terminator, a *recA–recX* message resulting from transcriptional read-through is detected at a level of 5–10% of the *recA* message (Pages et al., 2003). RecX is barely detected during vegetative growth, but robust expression of *recX* is detected after treating cells with DNA damaging agents (Stohl et al., 2003). The maximal *recX* expression is observed at a later time than maximal expression of RecA after UV irradiation (Courcelle et al., 2001). All these observations suggest that RecX is likely involved in the subtle regulation which helps shut off the SOS induction response.

Early studies have reported that DinI can destabilize the RecA-ssDNA filament at higher concentration (typically 50–100-fold above the natural RecA concentration) and

inhibit all activities of RecA (Voloshin et al., 2001; Yasuda et al., 1998). Therefore, DinI was initially thought to assist the SOS-induced cells return to a steady state. In contrast, recent research has shown that DinI is also able to stabilize the RecA-ssDNA filament so as to prevent its disassembly when present at concentrations that are stoichiometric with those of RecA or somewhat greater (Lusetti et al., 2004). Furthermore, DinI-mediated stabilization affects RecA-mediated UmuD cleavage rather than RecA-mediated ATP hydrolysis and LexA co-protease activities (Lusetti et al., 2004). It indicates that DinI could have a biological role in fine-tuning the activity of UmuD in order to limit SOS mutagenesis.

### 1.2.4. The role of DNA helicases and nucleases in SOS induction

A critical step in the SOS signalling mechanism is the production of a RecA-ssDNA filament, and this step requires DNA helicases and nucleases. Either the RecFOR or the RecBCD pathway is necessary for SOS induction after UV irradiation (Ivancic-Bace et al., 2006). In *E. coli*, SOS induction immediately after UV irradiation is dependent on the RecFOR pathway. The RecFOR pathway includes a DNA helicase RecQ, a nuclease RecJ, and the RecFOR complex which facilitates RecA loading (Lusetti et al., 2006). It has been suggested that the pathway may assist RecA binding to single-stranded gaps. RecQ was reported to be needed for fast degradation of the LexA repressor (Hishida et al., 2004). This observation leads to a model in which RecQ unwinds the template duplex in front of a stalled fork on the leading strand, and then switches over to the lagging strand to generate single stranded DNA on the lagging strand template, allowing formation of the RecA filament in the 5' to 3' direction for SOS induction (Heyer, 2004; Hishida et al., 2004).

RecBCD, also known as Exonuclease V, contains helicase, 5'-3' exonuclease, and RecA loading activities. It can directly load RecA on the processed double-strand break ends (Singleton et al., 2004). SOS induction after UV irradiation in *recFOR* mutants is not completely eliminated but is delayed and is dependent on the RecBCD enzyme (Hegde et al., 1995; Renzette et al., 2005; Thoms and Wackernagel, 1987; Whitby and Lloyd, 1995). Accordingly it has been proposed that SOS induction requires the RecBCD when the double-strand break ends appear later because of nucleotide excision repair and replication fork collapse after UV irradiation (Ivancic-Bace et al., 2006).

### 1.3. DNA damage induction in *S. cerevisiae*

The budding yeast *S. cerevisiae* is a simple unicellular eukaryotic organism, but it shares the complex internal cell structure of plants and animals. Much like *E. coli* as the model prokaryote, it is the most intensively studied eukaryotic model organism for the mechanisms of DNA damage repair and the regulation of DNA damage response. *S. cerevisiae* shares many of the technical advantages that permit rapid progress in the molecular genetics of prokaryotes, such as rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, a highly versatile DNA transformation system, numerous selective markers and easy gene manipulation (Sherman, 1997). The genome of *S. cerevisiae* was completely sequenced in 1996. It is composed of about 13,000,000 base pairs and 6,275 genes, and about 5,800 of these are believed to be true functional genes. It is estimated that 23% of yeast genes have homologs in human genome, and 30% of known genes involved in human diseases have yeast orthologs.

### 1.3.1. The DNA damage-inducible genes in *S. cerevisiae*

Like *E. coli*, when treating yeast cells with DNA damaging agents, numerous genes are transcriptionally induced. Many damage inducible genes in yeast were reported on the single-case based study. For example, in the study of DNA repair pathways, many genes in various yeast repair and tolerance pathways were identified to be DNA damage inducible (Friedberg et al., 2006). The *PHR1* gene involved in photoreactivation was reported to be induced by UV irradiation (Sebastian et al., 1990). The *MAG1* gene in BER is inducible by alkylating agents (Chen et al., 1990). The yeast NER genes *RAD2* (Robinson et al., 1986; Siede et al., 1989), *RAD7* (Jones et al., 1990), *RAD16* (Bang et al., 1995) and *RAD23* (Madura and Prakash, 1990) were characterized by a moderate degree of enhanced transcription after UV irradiation. In the recombination repair pathway, *RAD51*(Basile et al., 1992) and *RAD54* (Cole et al., 1987) are damage inducible. Both *RAD6* (Madura et al., 1990) and *RAD18* (Jones and Prakash, 1991) in PRR are induced by DNA-damaging treatments.

For genome-wide approaches, early studies used either differential plaque hybridization with cDNA from treated and untreated cells (McClanahan and McEntee, 1984) or a random *lacZ* fusion genomic library (Ruby and Szostak, 1985) to identify genes that are induced by DNA damaging agents. In McClanahan and McEntee's research, radiolabelled cDNA probes were generated from poly(A+) mRNA that was harvested from control, UV-treated, and 4NQO-treated cells. In addition, a λ library containing yeast genomic fragments was constructed and replica plates containing ~100 plaques each were made. When the probes were applied to the plaques, expression was determined by comparing the intensity of labelled probe bound between the control and treated samples. All together, they screened ~9000 genomic clones and identified four

DNA damage responsive (*DDR*) genes (McClanahan and McEntee, 1984). Ruby and Szastak constructed a random library in which yeast genomic DNA fragments were cloned into vectors bearing the *lacZ* coding sequence. Thus, each vector contains the fusion of "unknown" yeast genes (or segments) with the gene encoding β-galactosidase. Using nearly 8000 independent *lacZ* fusion clones, they compared β-gal activity over various DNA-damage inducing treatments (4-Nitroquinoline-*N*-oxide, UV, methotrexate, and gamma-irradiation). A total of 6 damage inducible (*DIN*) genes were identified (Ruby and Szostak, 1985).

Although these two early screening studies were significant to the field of damage induction, later studies suggested that these screens are biased for detecting genes with high induction ratios. High through-put analysis of global transcriptional responses to DNA damage has been made possible by the development of DNA microarray technologies. A DNA microarray (also known as DNA chip) is a high-density array of DNA spots that contain addressable complementary sequences to many or most genes in a genome. The chip is hybridized with fluorescently tagged nucleic acids, representative of expressed transcripts. Imaging and computational analysis are used to monitor relative transcript levels for thousands of genes simultaneously (Fry et al., 2005; Schena et al., 1995). Microarray studies provide a much deeper understanding of the DNA damage-induced transcription in budding yeast.

By using a DNA microarray, Jelinsky and Samson (Jelinsky and Samson, 1999) reported that 325 genes of 6,218 ORFs (5.2%) increase more than 4-fold in transcript level after exposing yeast cells to the alkylating agent MMS at 0.1% for 1 hour. These genes can be categorized into seven groups: DNA repair, detoxification, cell cycle, signal transduction, cell wall biogenesis, membrane transport and protein degradation.

Obviously, not only DNA repair genes and cell cycle regulation genes are regulated in response to DNA damage but also the genes involved in many other cellular processes. Later, the same laboratory determined the transcriptional continuum of yeast cells treated with different MMS doses and time courses (Jelinsky et al., 2000). More than 1000 genes were found to be up-regulated in response to MMS treatment. Interestingly, many of the genes induced by MMS treatment were also induced in response to the arrest of cells in the stationary phase. Thus there appears to be an overlap of responsive genes under two different stressful conditions, MMS exposure and stationary growth arrest. This might imply the existence of a general stress response pathway in budding yeast. Additionally, the transcriptional profiles were determined in yeast cells exposed to 5 other damaging agents: MNNG, BCNU, γ-ray, 4NQO, and the oxidizing agent *t*-BuOOH. Surprisingly, extensive differences were found between the transcriptional profiles induced by each of the six damaging agents. These differences may be explained by the fact that a microarray analysis just represents a simple snapshot of the transcriptional response to DNA damaging agents. A more extensive kinetic analysis will be helpful to find the overlap of responsive genes to different types of DNA damage.

In fact, studies on DNA damage-inducible genes imply that the damage induction in yeast seems not to be agent specific but rather occurs after treatment with a variety of DNA damaging agents. The *PHR1* gene is a noteworthy example whose transcription is induced by different DNA damage agents, including some that result in types of DNA damage which are not substrates for the protein. *PHR1* encodes a subunit of DNA photolyase which specifically removes UV-induced pyrimidine dimers (Sancar, 1985; Yasui and Langeveld, 1985). Since the Phr1 photolyase repairs exclusively pyrimidine dimers caused by UV irradiation, DNA lesions that are caused by agents like MMS are

obviously not substrates for the activated Phr1. However, the transcription of *PHR1* is induced not only by UV and the UV mimetic agent 4NQO but also by MMS, MNNG, bleomycin, and *cis*-diamminedichloroplatinin (Sancar, 2000; Sebastian et al., 1990). Another example is *MAG1*, which encodes the 3-methyl-adenine DNA glycosylase involved in protecting DNA against alkylating agents (Chen et al., 1989; Xiao et al., 1996). It is not surprising that the transcription of *MAG1* can be induced by MMS (Chen et al., 1990; Jia et al., 2002; Liu and Xiao, 1997). Nevertheless, it is also induced by hydroxyurea (HU) which results in depletion of deoxyribonucleotide pools and stalls at the replication forks (Liu and Xiao, 1997). Based on these observations, it seems likely that DNA damage induction is regulated by a global damage response pathway rather than a DNA lesion-specific pathway in budding yeast.

### 1.3.2. The pathways that regulate DNA-damage induction in *S. cerevisiae*

The transcriptional response to DNA damage in budding yeast shows that many genes are co-ordinately regulated in response to DNA damage. However, there is no evidence for a single regulon which is equivalent to the SOS regulon in *E. coli* for all DNA damage inducible genes. Therefore, it is probably safe to assume that there exist signal transduction pathways that detect DNA damage and convert damage signals into specific increases in the level of gene expression. According to the data from previous research, a putative model is proposed for signal transduction pathways: they consist of sensors, transducers and effectors (Bachant and Elledge, 1998). The sensors are proteins which initially sense the damaged DNA, and initiate the signaling response. Transducers can be activated by the DNA damage signal passed from the sensors, then amplify and relay the signal to the downstream effectors. The ultimate effectors execute the

regulation of transcription, and they are likely to be transcription factors which directly influence the levels of gene expression.

Sensors are the least known part in this model. So far, proteins which initially sense the DNA damage and initiate the signaling response are totally unknown. It is also unclear whether a limited number of sensors recognize the common DNA damage intermediates such as ssDNA (like RecA in the SOS response) or many different sensors detect each type of DNA lesion. Some proteins in yeast, such as the catalytic subunit of DNA polymerase ε (Pol2), share some of the properties expected for sensors, such as the potential to interact with DNA and the ability to activate the transcription of some damage-inducible genes (Navas et al., 1995). However, these data are obtained from single-case studies, and no published evidence indicates that they can activate the transcription of DNA damage inducible genes co-ordinately.

Conversely, the transducers are better characterized than sensors. Most of the transducers are protein kinases which can be activated by the DNA damage signal passed from the sensors, then amplify and relay the signal to the downstream effectors. In *S. cerevisiae*, protein kinases Mec1, Rad53 and Dun1 are necessary for the transcriptional response of many genes to DNA damage, and they appear to be transducers in the regulation network (Allen et al., 1994; Gasch et al., 2001; Kiser and Weinert, 1996; Zhou and Elledge, 1993). Meanwhile, Mec1, Rad53 and Dun1 are also DNA damage checkpoint kinases (Elledge, 1996). In addition, another DNA damage checkpoint protein Rad9 has also been reported to regulate the DNA damage induction of genes (Aboussekhra et al., 1996). Clearly, it suggests that the DNA damage checkpoint pathway contributes to the expression of genes that respond to DNA damage. The

relationship between DNA damage checkpoints and DNA damage induction will be discussed in the following section.

The first level of this signal transduction kinase cascade in budding yeast consists of Mec1 that belongs to the phosphatidylinositol 3-kinase family (PI-3K). Mec1 is required for the activation of Rad53 through PIKK-dependent phosphorylation of consensus PIKK sites within Rad53 (Ma et al., 2006; Pellicioli and Foiani, 2005). Activation of the serine/threonine protein kinase Rad53 is an essential intermediary step in yeast DNA damage responses which include delaying cell cycle progression, promoting repair processes, stabilization of stalled replication forks and inducing transcription (Branzei and Foiani, 2006). The protein kinase Dun1 is one of the identified targets of Rad53, and the activation of Dun1 requires phosphorylation by Rad53 (Chen et al., 2006). Dun1 was originally identified as a DNA-damage uninducible (dun) mutant defective in the induction of genes encoding ribonucleotide reductase (*RNR*) in response to DNA damage (Zhou and Elledge, 1993). Genome wide expression research showed that deletion of *DUN1* affected the expression of >1000 genes in response to both MMS and ionizing radiation, and the response in the *dun1Δ* mutant is largely the same as the response seen in the *mec1* mutant, suggesting that most of the Mec1-dependent effects on genomic expression are mediated by the downstream Dun1 kinase (Gasch et al., 2001). The mechanism employed by Dun1 for DNA damage induction seems to be quite diverse and awaits to be elucidated.

In the transcriptional response to DNA damage, the main mechanism employed is likely to be the regulation of transcription initiation. The downstream effectors are therefore expected to be transcription factors which directly influence transcription initiation. In a later section, selected examples will be introduced in which traditional

promoter analysis has provided insights into the molecular mechanisms of DNA damage-induced transcription.

### 1.3.3. DNA damage checkpoint pathway and DNA damage induction regulatory pathway

The DNA damage checkpoint mutants were first isolated by their phenotypes that fail to delay progression into mitosis after irradiation with x-rays (Rowley et al., 1992; Weinert and Hartwell, 1988; Weinert et al., 1994). These mutants showed radiation sensitivity, and the sensitivity could be largely restored by re-imposing a delay by other means. Hence the DNA damage checkpoint pathway was initially defined as a non-essential regulatory pathway that slows down or arrest cell cycle progression in response to DNA damage, allowing time for DNA repair (Hartwell and Weinert, 1989). However, this historic definition is inadequate to explain the function of this pathway completely. It is now suggested that the checkpoint pathway comprises a subroutine integrated into the larger DNA damage response that regulates a multifaceted response (Figure 1-1) (Zhou and Elledge, 2000). Besides arresting cell cycle progression, the DNA checkpoint pathway has been shown to promote DNA repair (Mills et al., 1999), control telomere length (Ritchie et al., 1999), activate transcription (Elledge, 1996), and trigger apoptosis in metazoan cells (Roos and Kaina, 2006). In a broad sense, DNA damage checkpoints regulate the DNA damage responses co-ordinately, including DNA damage induction. To avoid confusion on the definitions, Zhou and Elledge (Zhou and Elledge, 2000) refer to the entire pathway as "the DNA damage response pathway" and use "the checkpoint branch" for components specially involved in controlling cell-cycle progression.

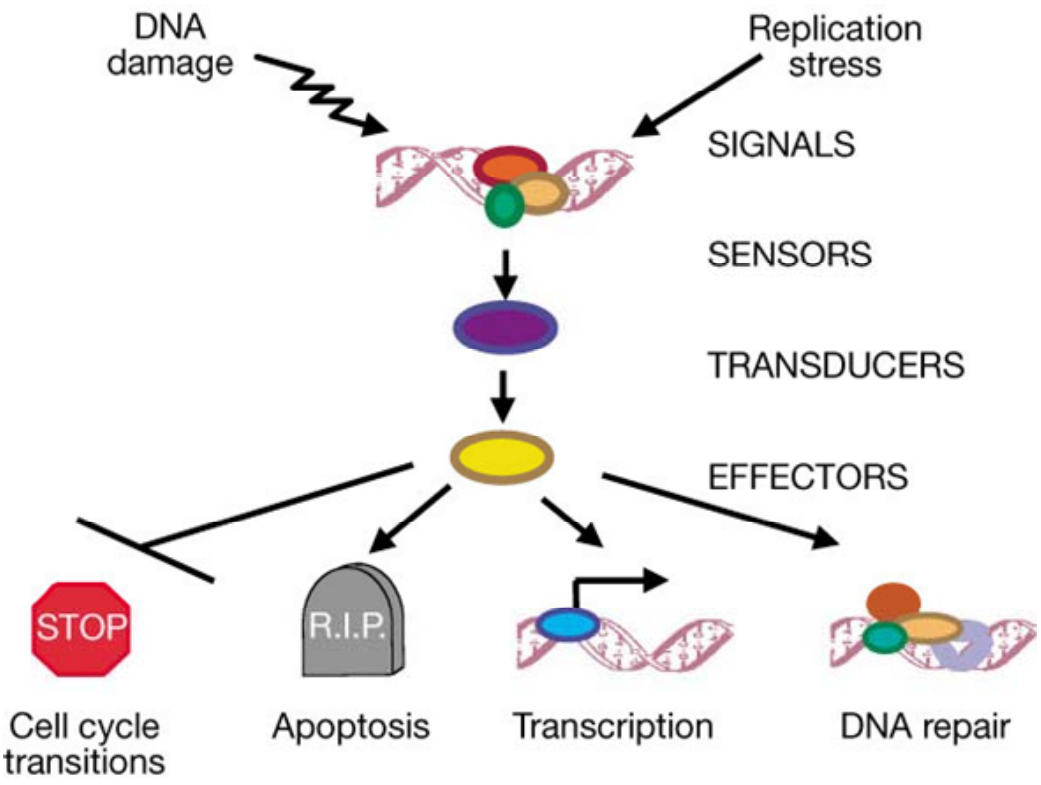



**Figure 1-1. The general outline of the DNA damage response signal transduction pathway.** Adapted from the review article "The DNA damage response: putting checkpoints in perspective" (Zhou and Elledge, 2000).

### 1.3.4. Molecular mechanisms of transcriptional regulation of yeast damage inducible genes

#### 1.3.4.1. Regulation of ribonucleotide reductase (*RNR*) genes

Regulation of *RNR* genes is the best-known example of eukaryotic transcriptional response to DNA damage. Ribonucleotide reductase is an enzyme converting nucleoside diphosphates (NDP) into deoxynucleoside diphosphates (dNDP), which represents the rate limiting step in the production of four dNTPs for DNA replication and repair (Elledge et al., 1993; Jordan and Reichard, 1998; Reichard, 1988). Any failure to control the amount and balance of dNTP pools will lead to mutagenesis or cell death. Ribonucleotide reductase is an $\alpha_2\beta_2$ tetramer, and four genes, *RNR1-4*, encode the subunits of budding yeast ribonucleotide reductase. *RNR1* and *RNR3* encode alpha (large) subunits, while *RNR2* and *RNR4* encode beta (small) subunits (Elledge and Davis, 1987; Elledge and Davis, 1990; Huang and Elledge, 1997). Expression of these genes are all inducible at the level of transcript accumulation by DNA damaging agents (Elledge and Davis, 1989; Elledge and Davis, 1990; Huang and Elledge, 1997).

The transcriptional level of *RNR3* gene is very low under normal conditions. However, when treating yeast cells with DNA damaging agents such as UV, MMS and 4-NQO, the transcriptional level of *RNR3* can be induced 100 to 500 fold (Elledge and Davis, 1990). In order to discover the mechanisms of induction, a series of mutants have been isolated that cause constitutive expression of *RNR3* (*crt* mutants) (Zhou and Elledge, 1992). These negative regulators of *RNR3* expression are divided into two groups: indirect regulators that result in endogenous DNA damage or a state of metabolic stress such as nucleotide depletion which induces the upregulation of *RNR3*, and direct

regulators involved in the entire signal transduction pathway, including transcription factors. The *CRT1*, *TUP1* (*CRT4*) and *SSN6* (*CRT8*) genes encode direct negative regulators binding to the *RNR3* promoter. A second screen was carried out in the same laboratory for the mutants that disrupt the ability of DNA damage to induce transcription of *RNR3*, and these genes were designated *DUN* for DNA-damage uninducible (Zhou and Elledge, 1993). The nonessential serine/threonine protein kinase Dun1 was isolated in this screen. Genetic analysis of *crt1Δ*, *tup1Δ* and *ssn6Δ* showed that these mutants were epistatic to the *dun1Δ* mutant, providing a strong genetic verification that *CRT1*, *TUP1* and *SSN6* function downstream of *DUN1* (Huang et al., 1998). Combined with the evidence that Mec1 and Rad53, as upstream kinases of Dun1, are necessary for the DNA damage induction of *RNR3* (Huang et al., 1998), the signal transduction regulation pathway for *RNR3* is clear.

In response to DNA damage or replication blocks, the Rad53 protein kinase is activated via a Mec1-dependent pathway, and activated Rad53 further phosphorylates the protein kinase Dun1. The Mec1-Rad53-Dun1 kinase cascade culminates in the phosphorylation of Crt1. Crt1 is a DNA-binding protein which binds to a 13-bp consensus sequence termed X-box. Multiple X-box related sequences of different strength can be found in the promoter region of *RNR3*, *RNR2* and *RNR4*. Crt1 is able to recruit the general repressor Tup1-Ssn6 complex to depress the transcription of *RNR* genes. In the presence of DNA damage or replication blocks, Crt1 is phosphorylated by Dun1 and loses the ability to bind to X-box, leading to transcriptional induction of *RNR* genes (Figure 1-2).

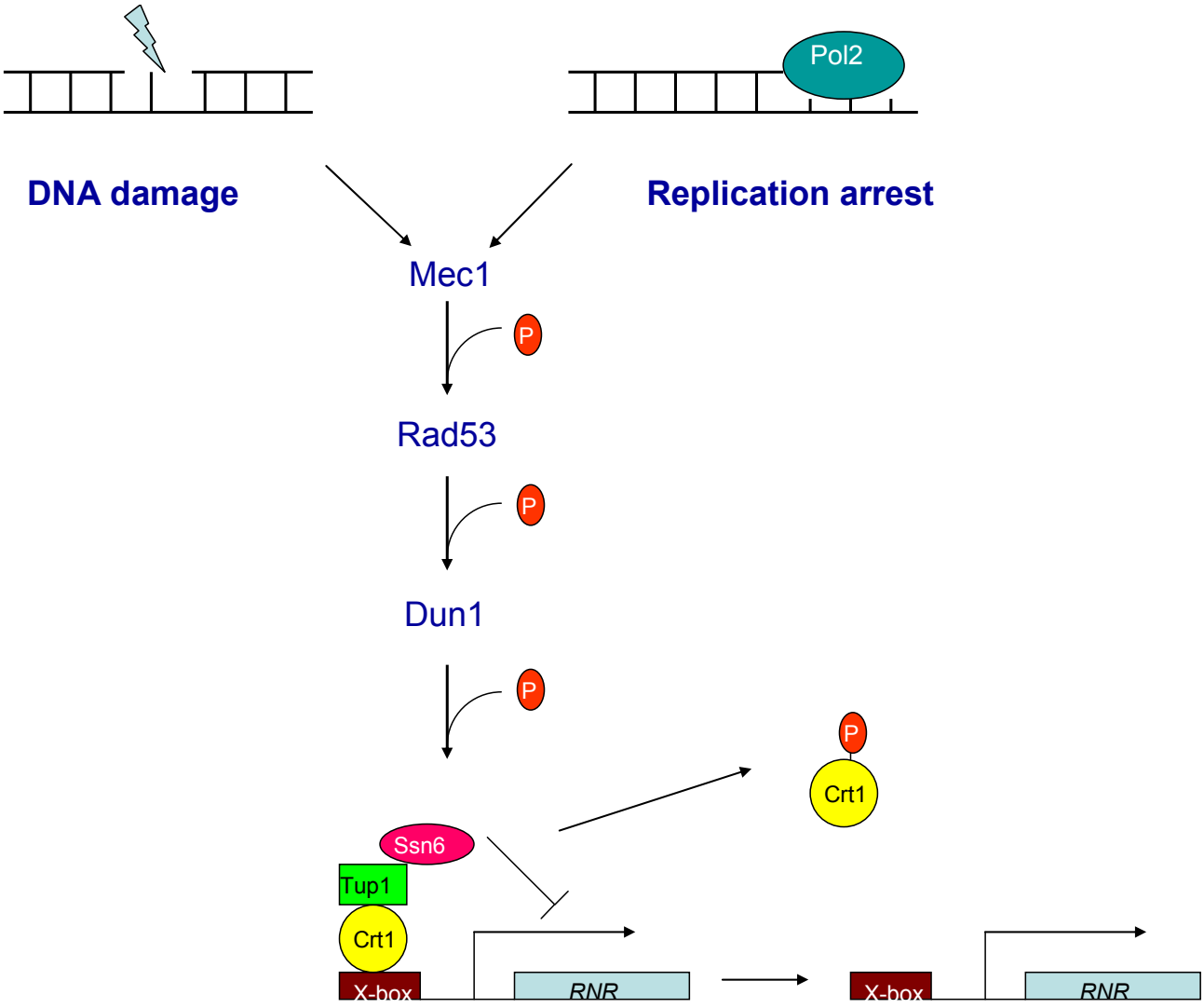



**Figure 1-2. Transcription control of *RNR* gene expression in budding yeast.** In response to DNA damage or replication arrest, checkpoint kinases Mec1, Rad53 and Dun1 are activated and phosphorylate the Crt1 repressor. The phosphorylated Crt1 loses affinity for its binding sequence, thus the transcription of *RNR* genes is increased.

Surprisingly, multiple X boxes were also identified in the *CRT1* promoter. Data from mutated X-boxes showed that they confer *CRT1*-dependent repression on *CRT1* itself (Huang et al., 1998). The expression level of *CRT1* is very low under normal growth conditions but is inducible by DNA damaging agents. Interestingly, the X-boxes in the *CRT1* promoter consist of one with very weak binding strength and one with very strong affinity to Crt1. Thus, it is likely that a weak induction of *CRT1* immediately occurs upon the presence of DNA damage which provides a buffer against spurious transcriptional activation of the pathway. Delayed full activation ensures a rapid restoration of the basal repressed state when the DNA damage is repaired (Huang et al., 1998).

Crt1 mediates repression by recruiting the general repressor Tup1-Ssn6 complex to *RNR* promoters via its N-terminus (Huang et al., 1998; Li and Reese, 2000). Tup1-Ssn6 recruitment establishes a nucleosomal array over the promoter of *RNR3* with a positioned nucleosome occupying the TATA box to block access by the general transcriptional machinery (Li and Reese, 2000; Li and Reese, 2001; Sharma et al., 2003). The depression of *RNR3* expression correlates with the disruption of nucleosome position. In response to DNA damage signals, the hyperphosphorylated Crt1 loses the ability to bind to the *RNR3* promoter, and the Tup1-Ssn6 complex is not recruited to the promoter region. Consequently, chromatin structure is remodelled, which increases the accessibility of DNA to transcription factors. The chromatin-remodeling of the *RNR3* promoter requires a number of general transcriptional factors, such as TBP-associated factors ($TAF_{IIS}$), and RNA polymerase II. Furthermore, the remodeling is also dependent on the SWI/SNF complex which possesses a DNA-stimulated ATPase activity and can destabilize histone-DNA interactions in an ATP-dependent manner (Sharma et al., 2003).

The preinitiation complex (PIC) components TFIID and polymerase assist in the recruitment of the SWI/SNF complex to the *RNR3* promoter, and retain the SWI/SNF complex at the remodeled promoter.

Recently, more regulatory factors were found to be involved in the damage induction of *RNR* genes. Wtm1 and Wtm2 have been reported to modulate expression of *RNR3* (Tringe et al., 2006). Moderate overexpression of both genes or high level expression of *WTM2* alone upregulates *RNR3-lacZ* in the absence of DNA damage. In response to HU and γ-ray, the expression level of *RNR3* is attenuated by 45% in *wtm2Δ* mutants, but not in *wtm1Δ* mutants. Wtm2 was found to directly associate with the *RNR3* promoter, and the association correlates with its ability to increase constitutive *RNR3* expression. So far, it is still unknown how Wtm2 increases *RNR3* transcription. From some observations, it is hinted that *WTM2* gene might enhance *RNR3* transcription by participating in chromatin remodeling (Tringe et al., 2006).

The *CCR4* gene encodes a component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening (Tucker et al., 2001). A strain defective in *CCR4* displayed particular sensitivity to the Rnr inhibitor HU (Woolstencroft et al., 2006). The *ccr4Δ dun1Δ* double mutants exhibited irreversible hypersensitivity to HU, and simultaneous overexpression of *RNR2*, *RNR3* and *RNR4* partially rescued the HU hypersensitivity of a *ccr4Δ dun1Δ* strain, suggesting that *CCR4* and *DUN1* function in different branches to regulate the activity of Rnr. It has been found that Ccr4 regulates *CRT1* mRNA poly(A) tail length and may subtly influence Crt1 protein abundance. Since *ccr4Δ* and *chk1Δ* exhibited epistasis in several genetic contexts, it is likely that Ccr4 and Chk1 act in the same pathway to overcome replication stress (Woolstencroft et al., 2006)

### 1.3.4.2. ***PHR1***

The budding yeast *PHR1* gene encodes a photolyase that repairs specifically and exclusively pyrimidine dimers which are the most frequent lesions introduced to DNA by UV irradiation (Sancar, 1985). The transcription of *PHR1* is induced by various DNA damaging agents. Three transcriptional regulatory elements have been defined within the *PHR1* promoter region: an upstream activating sequence (UAS), an upstream repressing sequence (URS) and an upstream essential sequence (UES) (Sancar et al., 1995). A 22-bp interrupted palindrome comprises $UAS_{PHRI}$, and it is responsible for 80-90% of basal and induced expression. It alone can activate transcription of a *CYC1* minimal promoter but does not confer damage responsiveness (Sancar et al., 1995). $URS_{PHR1}$ consists of a 39-bp region that includes a 22-bp palindrome. Deletion or specific mutations of $URS_{PHR1}$ increases basal level expression but decrease the induction ratio. It functions as a strong URS and confers a low level of damage inducibility when placed in the context of a heterologous gene (Sancar et al., 1995). The $UES_{PHR1}$ is required for efficient derepression when $URS_{PHR1}$ is present. Deletion of $URS_{PHR1}$ also eliminates the requirement for $UES_{PHR1}$ for transcriptional activation (Sancar et al., 1995).

Three proteins have been identified that regulate the expression of *PHR1* by binding to the upstream regulating elements. Ume6 is a bifunctional transcriptional regulator which is involved in several metabolic pathways (Strich et al., 1994). It is a positive regulator of *PHR1* transcription and binds specially to the $UAS_{PHR1}$ (Sweet et al., 1997). Multiple copies of Ume6 enhance expression of *PHR1*; however, the effect of deletion is growth phase dependent. Deletion of *UME6* reduces the expression of *PHR1* during vegetative growth, but only at a distinct phase (Sancar, 2000; Sweet et al., 1997). Rph1 and Gis1 are two DNA damage-responsive repressors of *PHR1* transcription, which

are 35% identical at the overall amino acid sequence level (Jang et al., 1999; Sancar, 2000). Both Rph1 and Gis1 contain two putative zinc fingers that are > 90% identical overall and completely identical in the DNA binding loop, and they regulate the DNA damage induction of *PHR1* through binding to $URS_{PHR1}$. Deletion of both *RPH1* and *GIS1* is required to fully derepress *PHR1* in the absence of damage, suggesting that they are functionally redundant. *In vitro* footprinting and binding competition studies indicate that the sequence $AG_4$ ($C_4T$) within the $URS_{PHR1}$ is the binding site for Rph1p and Gis1p (Jang et al., 1999).

Induction of *PHR1* expression is controlled by a DNA damage signal transduction pathway. Serine and threonine residues of Rph1 can be phosphorylated, and the phosphorylation of Rph1 increases in response to DNA damage. The DNA damage-induced phosphorylation requires DNA damage checkpoint proteins Rad9, Rad17, Mec1 and Rad53, indicating that the phosphorylation of Rph1 is under the control of the Mec1-Rad53 DNA damage checkpoint pathway (Kim et al., 2002). Furthermore, deletion of *DUN1*, *TEL1* and *CHK1* doesn't affect the phosphorylation of Rph1. These observations imply that DNA damage induction of *PHR1* is regulated by a potentially novel damage checkpoint that is distinct from the Mec1-Rad53-Dun1 protein kinase cascade implicated in the DNA damage-inducible transcription of *RNR* genes. Based on results of a co-immunoprecipitation (co-IP) assay, Rad53 seems not to physically interact with Rph1, indicating the existence of another kinase(s) in the Mec1-Rad53-Rph1 pathway (Kim et al., 2002). The identity of the kinase(s) is at present unknown.

### 1.3.4.3. *MAG1* and *DDI1*

*MAG1* encodes a 3-methyladenine DNA glycosylase that initiates BER to remove lethal lesions such as 3-methyladenine caused by alkylating agents. The transcription of *MAG1* is not only induced by DNA alkylating agents such as MMS, but also by UV and HU (Chen et al., 1990; Liu and Xiao, 1997). By analyzing the DNA sequence immediately upstream of *MAG1*, another DNA damage inducible gene, named *DDI1* for DNA Damage Inducible, was identified (Liu and Xiao, 1997). Like *MAG1*, *DDI1* is also induced by MMS, UV, 4NQO and HU. Furthermore, both genes require a similar dosage for the maximum induction, and the fold of induction is similar (Liu and Xiao, 1997).

*MAG1* and *DDI1* lay in a head-to-head configuration and are transcribed divergently. The expression of *MAG1* and *DDI1* is controlled by two functionally opposite regulatory elements, UAS and URS, through an antagonistic mechanism (Liu and Xiao, 1997). A $UAS_{MAG1}$ and a $URS_{MAG1}$ have been identified in the promoter region of *MAG1* (Xiao et al., 1993). The expression of *DDI1* was reported to be negatively regulated by a $URS_{DDI1}$ in its promoter region (Liu and Xiao, 1997). The intergenic region between *MAG1* and *DDI1* also contains *cis*-acting elements that co-regulate the expression of both genes. In the common promoter region, $UAS_{DM}$, which contains two 8-bp tandem repeat sequences 5'-GGTGGCGA-3', is required for the bidirectional expression of *MAG1* and *DDI1* (Liu and Xiao, 1997). With a yeast one-hybrid screen using the $UAS_{DM}$ as a bait, a transcriptional activator called Pdr3 was isolated (Zhu and Xiao, 2004). Deletion of *PDR3* reduced both basal-level expression and DNA damage induction of *MAG1* and *DDI1*. In addition, deletion of *PDR3* does not further affect *MAG1* and *DDI1* expression if $UAS_{DM}$ is deleted, indicating that $UAS_{DM}$ is indeed the target of Pdr3 activation (Zhu and Xiao, 2004). Furthermore, another

transcriptional activator Rpn4 was shown to be required for both *MAG1* and *DDI1* expression (Jelinsky et al., 2000). However, Rpn4 does not appear to bind $UAS_{DM}$. Moreover, deletion of *RPN4* does not alter *MAG1* and *DDI1* expression in the *pdr3* mutant cells, suggesting *RPN4* acts upstream of *PDR3* (Zhu and Xiao, 2004). Deletion of *PDR3* and *RPN4* has no effect on the basal-level and DNA damage-induced expression of *PHR1*, *RNR2* and *RNR3*. Meanwhile, the regulators of *RNR* genes Crt1, Tup1/Ssn6 and the regulators of *PHR1* gene Rph1 and Gis1 are not involved in the control of *MAG1* expression (Zhu and Xiao, 2001). Hence, it seems that all three sets of well-studied yeast damage-inducible genes (*RNR*, *PHR1* and *MAG1-DDI1*) have distinct regulators and that the regulatory mechanisms are also different from each other.

The DNA damage induction of *MAG1* and *DDI1* is also controlled by DNA damage checkpoints. Mutation of *POL2*, *MEC1* or *DUN1* reduces the DNA damage induction of *MAG1* (Zhu and Xiao, 1998; Zhu and Xiao, 2001). Thus, it suggests that *MAG1* is regulated by the *POL2-MEC1-RAD53-DUN1* checkpoint pathway. However, in both *dun1Δ* mutant and *mec1Δ* mutant, *DDI1* are fully induced by MMS treatment. While in *pds1Δ sad1-1* (*rad53*) and *pds1Δ dun1Δ* double mutants, the DNA damage-dependent induction of *DDI1* is significantly inhibited compared to the respective single mutants (Zhu and Xiao, 2001). *PDS1* encodes an anaphase inhibitor and functions in DNA damage checkpoints. *PDS1* and *RAD53-DUN1* may form two parallel branches in DNA damage checkpoints (Gardner et al., 1999; Schollaert et al., 2004). This suggests that the *PDS1* and *MEC1-RAD53-DUN1* checkpoint pathways may function redundantly in the control of *DDI1* expression (Zhu and Xiao, 2001).

## 1.4. DNA damage induction in mammalian cells

Compared with budding yeast, the transcriptional responses to DNA damaging agents in mammalian cells are more complex. The complexity is not only due to the larger and more intricate genomes of mammalian cells compared to yeast cells, but also to a greater range of possible downstream responses (Friedberg et al., 2006). Firstly, the presence of a damaged cell in a tissue may initiate an extracellular communication of damage response. Secondly, mammalian genes that are activated in response to DNA damage include those genes involved in DNA repair and repair-associated processes, as well as genes encoding secreted growth factors, growth factor receptors, protective cytoplasmic enzymes, and proteins normally associated with tissue injury and inflammation (Eckardt-Schupp and Klaus, 1999; Friedberg et al., 2006; Herrlich et al., 1992; Kastan et al., 1992; Keyse, 1993; Weichselbaum et al., 1991). Thirdly, if the damage is too severe to be fixed, mammalian cells will initiate programmed cell death, namely, apoptosis or autophagy. Conversely, budding yeast as a unicellular lower eukaryote does not undergo apoptosis, or only in a rudimentary form (Ludovico et al., 2005; Weinberger et al., 2003). A number of genes involved in apoptosis are transcriptionally regulated in response to the cell death signal.

### 1.4.1. The transcriptional response to DNA damage in mammalian cells

Although DNA damage induction in mammalian cells is more complicated than that in budding yeast, the pathways involved in the regulation of DNA damage induction in yeast and mammalian cells are still evolutionarily conserved. Like yeast, the well-conserved DNA damage checkpoint pathways play a role in DNA damage induction in mammalian cells. The regulatory pathways are also signal transduction pathways

consisting of sensors, transducers and effectors. In these signal transduction pathways, the identities of the sensors are not yet known, whereas much is known about the signal transducers. ATM and ATR kinases, CHK1 and CHK2 kinases are well-documented transducers.

#### 1.4.1.1. ATM and ATR

The ATM (Ataxia-telangiectasia mutated) gene was identified as the gene mutated in the human genomic instability syndrome *ataxia telangiectasia* (AT) (Savitsky et al., 1995). Both ATM and ATR (ATM and Rad3-related) belong to the PI-3K family, and they exhibit sequence similarity to Tel1 and Mec1 in the budding yeast. These two proteins play a key role in mammalian DNA damage checkpoints. The ATM and ATR associated kinase activities are enhanced in response to DNA damage. ATM responds primarily to DSBs induced by IR, while ATR plays important roles in response to damages caused by UV and HU(Abraham, 2001; Kurz and Lees-Miller, 2004; Yang et al., 2003). However, there is no strict division between the signals for the two protein kinases, since ATM also functions in some UV responses (Hannan et al., 2002; Yang et al., 2003). So far, it is still not very clear how these two kinases are activated by DNA damage. Several mechanisms have been proposed for the activation: a) activation through interaction with damaged DNA, b) activation through interaction with proteins of the DNA repair complex, c) a combination of both (Wahl and Carr, 2001; Yang et al., 2004). Based on some observations, it has been speculated that ATM might be activated through interactions with an altered chromatin structure due to strand breaks rather than direct binding to DSB (Bakkenist and Kastan, 2003; Bartek and Lukas, 2003). The activation of ATR might be different from that of ATM. ATR binds to UV-damaged

DNA with higher affinity than to undamaged DNA (Unsal-Kacmaz et al., 2002). In addition, damaged DNA stimulates the kinase activity of ATR to a significantly higher level than undamaged DNA. Nevertheless, the mechanism for ATR activation remains to be elucidated.

Following DNA damage, many proteins aggregate at the damage sites, thus forming nuclear "foci". ATM and ATR can be found in these foci within 5 minutes of the damage occurring. Other proteins found in the foci include DNA repair proteins, chromatin structure proteins and transcriptional factors (Yang et al., 2003). This suggests that the central role of ATM and ATR is DNA damage response.

#### 1.4.1.2. CHK1 and CHK2 kinases

CHK1 and CHK2 are two protein kinases which act downstream of ATM and ATR in the damage checkpoint pathways. CHK2 is the homolog of Rad53 in budding yeast, and CHK1 shares homology with the budding yeast Chk1. In human and other vertebrates, CHK1 and CHK2 appear to have unique functions but show a high degree of cross-talk and connectivity. CHK2 is rapidly phosphorylated and activated in response to replication blocks and DNA damage in an ATM dependent manner (Matsuoka et al., 1998). After exposure to ionizing radiation, ATM directly phosphorylates CHK2 at Thr68 and the residues in the ST/QT cluster, and induces autophosphorylation in CHK2 (Ahn et al., 2000; Lee and Chung, 2001; Ward et al., 2001). Moreover, phosphorylation of CHK2 by ATM occurred exclusively at the DSB sites. Unlike the case for other DNA repair proteins, the phosphorylated CHK2 is able to move rapidly through the nucleus. Forced immobilization of CHK2 impairs its stimulating effect on p53-dependent transcription (Lukas et al., 2003). It indicates that CHK2 migrates from the DNA

damage regions to its physiological targets after phosphorylation by ATM. Human CHK1 is phosphorylated at Ser317 and Ser345 in response to UV irradiation or HU treatment. The phosphorylation is inhibited in ATR-deficient cells. Mutants of CHK1 containing S317A and S345A are poorly activated in response to replication blocks and DNA damaging agents (Zhao and Piwnica-Worms, 2001). In addition, phosphorylation of CHK1 on Ser-317 in response to IR is markedly reduced in the cells lack of ATM, and the expression of ATM can correct this defect (Gatei et al., 2003). In contrast, the phosphorylation of CHK1 in response to HU and UV is ATM-independent.

### 1.4.2. Effectors in the DNA damage induction

Transcription factors are the ultimate effectors which directly influence transcription in signal transduction pathways. The specific activation of a number of transcription factors has been observed in mammalian cells following UV irradiation or ionizing radiation.

#### 1.4.2.1. The p53 transcription factor

The p53 protein is known as a "guardian of the genome" because of its crucial role in mediating the cellular responses to genotoxic stress (Levine, 1997). It is a sequence-specific transcriptional factor which functions as a potent tumor suppressor. p53 both positively and negatively regulates the expression and activity of many genes involved in cell cycle arrest, DNA synthesis and repair as well as apoptosis. Inactivating mutations in the *p53* gene are found in 50% of cancers, while the other half are thought to contain mutations in components associated with the p53 pathway (Coutts and La Thangue, 2006; Friedberg et al., 2006). Germline mutations of *p53* cause Li-Fraumeni

syndrome (LFS) which predisposes patients to spontaneous tumor formation (Malkin, 1994).

The p53 protein forms a homo-tetramer when it functions as a transcription factor, and a 53-amino-acid carboxy-terminal domain is responsible for the tetramerization. Its DNA binding domain is mapped to residues 102-292 (Pavletich et al., 1993) (Figure 1-3). The consensus binding site of p53 shows a striking internal symmetry, consisting of two copies of the 10-bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs. The binding of p53 requires both copies of the motif, and any subtle alterations in the motif cause loss of affinity for p53 (el-Deiry et al., 1992).

#### 1.4.2.1.1. The activation of p53

Under normal conditions, p53 is kept in a latent inactive state and retains low protein levels through the regulation of protein stability. Following DNA damage and other stresses, such as ribonucleotide depletion (Linke et al., 1996), p53 is activated and undergoes a significant increase in protein stability primarily by post-translational modifications.

HDM2, the human homolog of murine double minute 2 (Mdm2) oncoprotein, is a key regulator of p53 in human cells (Bottger et al., 1997; Brooks and Gu, 2006; Coutts and La Thangue, 2006). Mdm2 binds to the N-terminal region of p53 to block the interactions between p53 and transcriptional co-activators and ubiquitinate p53. Accordingly, the transcriptional activity of p53 is repressed, and ubiquitinated p53 is targeted for proteosomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). In response to DNA damage, ATM and ATR are activated and directly phosphorylate p53

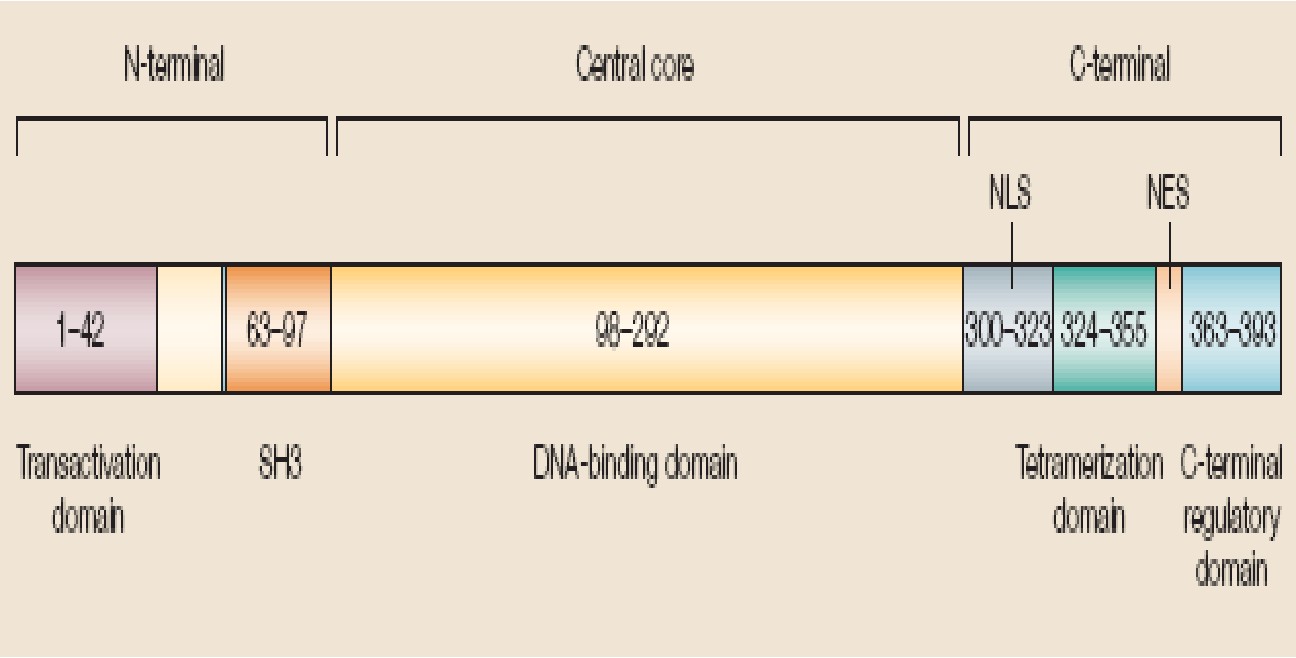



**Figure 1-3. The functional domains of human p53.** The transactivation domain and the Src homology-3-like (SH3) domain are located at the N-terminus of p53. The central core contains the DNA-binding domain, and the C-terminus contains nuclear localization and export signals, a regulatory domain and the tetramerization domain. Numbers indicate residue number. Adapted from (Bode and Dong, 2004).

on Ser15 which is in the transactivation domain (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Their downstream kinases CHK1 and CHK2 phosphorylate p53 on Ser20 which is within the Mdm2-binding region (Hirao et al., 2000). Furthermore, some other distinct protein kinases phosphorylate p53 at different serine or threonine sites at the N-terminus (Bode and Dong, 2004). Phosphorylation of p53 at the N-terminus disrupts the binding of Mdm2 so as to stabilize the protein. The phosphorylated p53 also shows increased binding activity to its specific DNA sequence and the ability to recruit co-activators (Hupp and Lane, 1994; Lambert et al., 1998). In addition, phosphorylation promotes phosphorylation and acetylation on the C-terminus of p53. So far, 17 phosphorylation sites have been detected in human p53 in response to DNA damage induced by ionizing radiation or UV irradiation (Bode and Dong, 2004). The presence of multiple phosphorylation sites may help fine tune the activation of p53. Interestingly, the transcription of *mdm2* is regulated by p53, thus an autoregulatory feedback loop is involved in the tight regulation of p53 (Barak et al., 1993). Moreover, HDM2 is rapidly phosphorylated on Ser395 by ATM after ionizing radiation treatment. The phosphorylated HDM2 may be less capable of mediating degradation of p53 (Maya et al., 2001).

CBP (cAMP response element-binding protein) and p300 are histone acetylases that not only acetylate chromatin but also many transcription factors, including p53 (Chan and La Thangue, 2001). In response to DNA damage, the phosphorylation of Ser15 in p53 enhances the binding of p300/CBP and pCAF (p300/CBP-associated factor) to the N-terminus of p53 so that they acetylate the C terminus of p53 on the lysine residues (Lambert et al., 1998; Liu et al., 1999). These lysine residues are also targets of Mdm2 ubiquitination, suggesting a potential competition between acetylation and

ubiquitination. Hence acetylation of p53 by p300/CBP and pCAF might negatively affect Mdm2-mediated ubiquitination and increase p53 stability. Furthermore, CBP/p300 also acetylates Mdm2 in its C-terminal RING-finger domain (Wang et al., 2004). This modification is likely to impair E3 ligase activity of Mdm2, thus it serves as an additional mechanism for the inactivation of Mdm2. Additionally, p300/CBP bound to p53 acetylates nucleosomes in p53 response elements, resulting in chromatin modifications required for p53 transcriptional activation (Espinosa and Emerson, 2001).

It is well known that genotoxic stresses, such as ionizing radiation and UV irradiation, result in the nuclear accumulation of p53. Stress-induced nuclear accumulation is probably due to an inhibition of nuclear export. As discussed before, p53 binds to its response elements as a homo-tetramer. In its C-terminal tetramerization domain, a highly conserved nuclear export sequence (NES) is responsible for the export of p53 from the nucleus (Stommel et al., 1999). Mutations in the NES lead to a predominantly nuclear localization of p53. Meanwhile, these mutations also prevent the formation of p53 tetramers. Crystal structure analysis reveals that NES is exposed and should be a suitable substrate for an export receptor when p53 is monomeric or dimeric, but is buried in p53 tetramers (Stommel et al., 1999). These observations suggest a mechanism for the tight connection between p53 structure and function following DNA damage. The enhanced stability of p53 improves the formation of p53 tetramers, thus increasing both p53 nuclear accumulation and binding to response elements (Wahl and Carr, 2001).

#### 1.4.2.1.2. p53-mediated gene expression

Since p53 is a sequence-specific transcriptional factor, the transcriptional transactivation might be its primary function in DNA damage responses. Both the DNA-binding domain and the transactivation domain are indispensable for its tumor suppression function. Mice with an allele encoding changes at Leu25 and Trp26 in p53, which is known to be essential for transcriptional transactivation, exhibit the same predisposition to tumor formation as the isogenic mice with a p53-null allele (Jimenez et al., 2000). Inactivating mutations in the DNA binding domain of p53 has been shown to be one of the main causes that lead to tumor formation (Cho et al., 1994; Pietenpol et al., 1994).

Two strategies has been carried out to identify biologically important genes which are transcriptionally regulated by p53 (Nakamura, 2004). The first approach is the differential display method and cDNA microarray analysis. The data from microarray analysis show that the p53-mediated transcription vary widely among cell lines and individuals (Burns and El-Deiry, 2003; Friedberg et al., 2006; Maxwell and Davis, 2000; Mirza et al., 2003; Okada et al., 2003; Sax et al., 2003; Zhao et al., 2000). From these studies, p53 appears to regulate the transcription of more than 1000 genes in response to DNA damage. The second approach is based on the idea that p53 is able to directly regulate genes through binding p53 responsive elements in promoter regions or within introns. According to GenBank annotation and a computationally derived transcript map, a p53 target gene database of human genes shows that the promoters of 4,852 genes contain at least one p53 consensus binding sequence (Wang et al., 2001). Recently, the promoter regions bound to p53 were screened during DNA damage through chromatin immunoprecipitation (ChIP) coupled with CpG island (CGI) microarray analysis, (Krieg

et al., 2006). The screen identified promoters of 199 genes which are directly bound by p53 in response to DNA damage. Binding of p53 to these target genes is partially dependent on a functional DNA binding domain, and the expression of these genes varies a great deal in response to stress (Krieg et al., 2006).

Taken together, the following 6 groups of genes are transcriptionally regulated by p53: (i) genes with a role in DNA repair, such as the ribonucleotide reductase gene *p53R2* (Tanaka et al., 2000), NER genes *GADD45*, *XPC* and *XPA* (Adimoolam and Ford, 2002; Hwang et al., 1999; Tan and Chu, 2002), MMR genes *hMSH2* (Scherer et al., 2000) and *PCNA* (Krieg et al., 2006; Xu and Morris, 1999); (ii) genes regulating cell cycle progression, including *p21* (Li et al., 1994), *GADD45* (growth arrest and DNA damage inducible) (Kastan et al., 1992), *hCDC4b* (Kimura et al., 2003) , *cyclin K* (Mori et al., 2002) and *p53RFP* (p53-inducible RING-finger protein) (Ng et al., 2003); (iii) genes involved in oxidative stress, such as the *ALDH4* gene (aldehyde dehydrogenase 4) (Nakamura, 2004); (iv) genes that function in apoptosis, such as genes encoding death domain proteins Bax, PUMA and Noxa; *BCL2* family members; *FAS*, *KILLER/DR5* and *PIDD*, genes in the death receptor pathway; *PERP* gene encoding a membrane protein (p53 apoptosis effector related to PMP-22); and *APAF-1* gene (apoptosis protease activator apoptotic activating factor 1) (Shen and White, 2001; Slee et al., 2004; Wahl and Carr, 2001); (v) genes influencing the extra-cellular matrix or cytoskeleton, such as the collagen genes (Bian and Sun, 1997; Eizenberg and Oren, 1991; Zhao et al., 2000) and cytokeratin genes (Cui and Donehower, 2000; Kazachkov et al., 1996; Zhao et al., 2000); (vi) genes inhibiting angiogenesis, e.g. *TSP1* (thrombospondin 1) (Dameron et al., 1994) and *BAI1* (brain- specific angiogenesis inhibitor 1) (Nishimori et al., 1997).

#### 1.4.2.1.3. Molecular mechanisms for transcriptional activation by p53

p53 may promote transcription by three possible mechanisms. First, p53 binding to its responsive elements recruits chromatin remodeling factors, histone transacetylases and methyltransferases to facilitate the initiation of transcription. For example, several subunits of the human SWI/SNF complex bind to p53, and the SWI/SNF complex is necessary for p53-driven transcriptional activation (Lee et al., 2002). As mentioned before, histone transacetylases p300/CBP and pCAF bind to the N-terminus of p53 and further acetylate nucleosomes in the promoter region, resulting in chromatin modifications required for transcriptional activation. The involvement of protein arginine methyltransferases PRMT1 and CARM1 in p53 function have been demonstrated (An et al., 2004). These coactivators directly interact with p53 and modify cognate histone substrates to promote the initiation of transcription. Secondly, p53 stimulates transcription via enhancing the recruitment of the basal transcription factors TFIIA and TFIID on the promoter region. p53 induces a conformational change over the promoter region to form a more stable and active TFIIA-TFIID-DNA complex, which may lead to enhanced assembly of the rest of the factors of the transcription machinery (Xing et al., 2001). Thirdly, p53 may directly interact with the components in the mediator complex to facilitate the formation of a pre-initiation complex (Gu et al., 1999; Zhang et al., 2005).

### 1.4.2.2. The AP-1 transcription factor complex

AP-1 (activator protein-1) is a complex of sequence-specific transcriptional activators consisting of the proteins of the FOS and JUN families (Angel and Karin, 1991). All three JUN variants (c-Jun, JunB and JunD) and four FOS variants (c-Fos, FosB, Fra-1 and Fra-2) belong to the basic leucine zipper (bZIP) superfamily of DNA

binding proteins, and they are homologous to transcription factor Gcn4 in budding yeast. FOS and JUN make up AP-1 by forming a homodimer, a heterodimer or a complex with closely related transcription activators such as ATF-2 (activating transcription factor 2) (Angel and Karin, 1991). In addition, Maf (musculoaponeurotic fibrosarcoma oncogene family ) proteins can heterodimerize with JUN or FOS to make up AP-1 (Kataoka et al., 1994; Swaroop et al., 1992). The heterodimer of c-Jun with c-Fos exhibit the highest transcriptional capacity since this complex is the most stable dimer, although this varies depending on promoter context (Chen et al., 1998; Kouzarides and Ziff, 1988). The AP-1 transcription factor complex is highly responsive to genotoxic stress. Following exposure of mammalian cells to different DNA damaging agents, the DNA-binding and transcription activities of AP-1 are significantly elevated, resulting in the enhanced transcription of at least 250 genes involved in DNA repair, cell cycle regulation and tumor progression (Davis et al., 1998; Frame et al., 1991; Hayakawa et al., 2004; Sahijdak et al., 1994).

The activity of AP-1 is regulated at two levels: synthesis of new AP-1 components or activation of a pre-existing AP-1 complex through post-translational modification (Karin, 1995). The transcription of most genes encoding AP-1 components is rapidly activated within 15 to 20 minutes after UV irradiation so that *FOS* and *JUN* are described as "immediate-early-response genes" (Treisman, 1992). AP-1 can bind to two *cis*-elements (nt. -190 to -180 and -71 to -64) in the *c-jun* promoter to activate the transcription of *c-jun* itself after UV treatment (Devary et al., 1991). The regulation of *c-fos* transcription is more complex. Several *cis*-elements mediate *c-fos* induction in response to DNA damage, including the cAMP responsive element (CRE) recognized by

AP-1 (Treisman, 1992). All these observations indicate a positive-feedback loop on the transcription level for the regulation of AP-1 activity.

Like p53, the activity of AP-1 is regulated by post-translational modification. Phosphorylation of Ser63 and Ser73 in the transactivation domain of JUN by JUN N-terminal kinases (JNK) potentiates its ability to activate transcription as either a homodimer or a heterodimer with FOS (Deng and Karin, 1994; Derijard et al., 1994; Pulverer et al., 1991). ATF can be phosphorylated on Thr69 and Thr71 within its N-terminal transactivation domain by JNK as well, and this phosphorylation upregulates the transcriptional activity of ATF (Gupta et al., 1995).

Multiple signal transduction pathways contribute to the phosphorylation of AP-1. Ionizing radiation induces the phosphorylation of c-Jun in normal fibroblasts but not in AT fibroblasts, suggesting that ATM plays a role in the phosphorylation of JUN (Lee et al., 1998). JNK can be activated through phosphorylation by MEKK1 (MEK kinase kinase 1)-MKK4 (MAP kinase kinase) kinase cascade. MEKK1 in turn is activated by ABL which is a tyrosine kinase stimulated by ionizing radiation (Kharbanda et al., 2000; Kharbanda et al., 1995). ABL is a downstream target of ATM, and phosphorylation at Ser465 by ATM is required for ABL kinase activity in the cellular response to ionizing radiation (Baskaran et al., 1997). This ATM –mediated pathway is not activated by UV irradiation.

Following UV irradiation, a signal transduction pathway originating from the cell membrane regulates the phosphorylation of AP-1. UV irradiation activates the growth factor and cytokine receptors on the cell membrane, e.g. epidermal growth factor (EGF) receptor, interleukin (IL)-1 receptor, insulin receptor, platelet derived growth factor (PDGF) receptor and tumor necrosis factor-α (TNF-α) receptor, resulting in clustering

and internalization of these receptors (Rittie and Fisher, 2002; Rosette and Karin, 1996). Activation of cell surface cytokine and growth factor receptors subsequently activate small GTP-binding protein family members Ras, Rac, and Cdc42 to trigger downstream ERK, JNK and p38 MAPK signalling pathways. JUN and ATF are subject to phosphorylation by JNK, ERK and p38 MAPK.

#### 1.4.2.3. The E2F transcription factor family

E2F transcription factors normally are heterodimers consisting of a subunit of the E2F family and a subunit of the DRTF1 protein (DP) family. In mammals, the E2F family includes eight proteins (E2F1-8), and there are three proteins (DP1, DP2/3 and DP4) in the DP family (DeGregori and Johnson, 2006; Du and Pogoriler, 2006). E2F1-6 require dimerization with one of three DP proteins to form functional transcription factors, whereas recently identified E2F7 and E2F8 (in mouse) exhibit little homology to their traditional counterparts and function independently of DP proteins (Christensen et al., 2005; de Bruin et al., 2003; Logan et al., 2005; Maiti et al., 2005; Milton et al., 2006; Rogers et al., 1996; Wu et al., 1995). All E2F1-6 and three DP proteins possess a conserved DNA binding domain and one dimerization domain. Conserved domains for transcriptional activation and pocket protein binding are present only in E2F1–E2F5. Rb (Retinoblastoma) and/or the other pocket proteins, p107 and p130, can bind to E2F1-5 and block the ability of an E2F-DP heterodimer to activate transcription.

Early studies showed that E2F transcription factors plays a critical role in organizing the G1/S transition by regulating the transcription of genes required for entry into S phase (La Thangue, 1994; Ohtani et al., 1995). Recently, global ChIP–chip assays of E2F4 and E2F1 revealed that E2F transcription factors directly regulate genes of seven

functional groups: cell cycle regulation, DNA replication, DNA repair, DNA damage and $G_2$/M checkpoints, chromosome transactions, and mitotic regulation (Ren et al., 2002). DNA repair genes regulated by E2F are involved in the full spectrum of repair processes, including MMR(*MSH2*, *MLH1*), BER(*UNG*), NER(*RPA3*), homologous recombination (*RAD51* and *RAD54*) and NHEJ(DNA-dependent protein kinase). Additionally, a linkage has been observed between the processes of DNA replication and repair in mammalian cells, indicating that their expression is co-regulated through E2F (Ren et al., 2002).

Like p53, E2F1 is stabilized by UV irradiation and ionizing radiation (Blattner et al., 1999). The degradation of E2F1 is mediated by the ubiquitin proteasome pathway (Campanero and Flemington, 1997). Similar to p53, the ubiquitination of E2F1 also depends on MDM2. Moreover, MDM2 interacts with E2F-1 via the same domain that it interacts with p53. Therefore it suggests that E2F-1 is upregulated in a manner similar to p53 in response to DNA damage and Mdm2 appears to play a major role in this pathway (Blattner et al., 1999). In response to DNA damage, E2F-1 is targeted and phosphorylated by ATM/ATR and CHK2 (Lin et al., 2001; Stevens et al., 2003). Both ATM and ATR can directly phosphorylate E2F1 at Ser31 (Lin et al., 2001). The phosphorylation increases protein stabilization, resulting in the accumulation of E2F1. The DNA damage-induced accumulation of E2F is blocked in either E2F Ser31Ala mutant or ATM kinase dead mutant (Lin et al., 2001). After treating cells with DNA damaging agents, CHK2 can also phosphorylate E2F-1 at the Ser364 residue (Stevens et al., 2003). Phosphorylation by CHK2 increases the E2F-1 level by an extended half-life of E2F. Phosphorylation on Ser364 possibly decreases the physical association between MDM2 and E2F-1 since Ser364 is positioned close to the MDM2 binding domain in E2F1. By comparing the effects of E2F-1 wild type with E2F-1 Ser364Ala mutant on

E2F-responsive promoters, the transcription activity of an E2F-1 point mutant is significantly lower than that of E2F-1 wild type, even though protein levels were similar (Stevens et al., 2003). It seems that phosphorylation of E2F-1 by CHK1 is functionally important for both the stability of E2F-1 and the ability of E2F-1 to activate transcription (Stevens et al., 2003). Most interestingly, several E2F binding sites exist in the *CHK1* promoter. Overexpression of E2F-1 can induce the expression of *CHK1*, implying a positive feedback loop in the DNA damage response (Carrassa et al., 2003).

Similar to p53, E2F-1 is also regulated by acetylation. In response to DNA damage, E2F-1 is acetylated at Lys117, Lys121 and Lys125 by the p300/CBP-associated factor P/CAF and to a less extent by p300/CBP itself (Martinez-Balbas et al., 2000). Acetylation of E2F-1 by P/CAF increases its DNA-binding ability, transcriptional activation capacity and protein half-life (Martinez-Balbas et al., 2000). Moreover, both acetylation and phosphorylation of Ser31 by ATM/ATR are required for efficient recruitment of E2F-1 to its target promoter after DNA damage (Pediconi et al., 2003). Thus, E2F-1 acetylation seems to be connected with phosphorylation in directing E2F1 to the promoters of target genes in response to DNA damage.

## 1.5. The transcriptional repression in the DNA damage response

Compared with genes whose expression is up-regulated in response to DNA damage, the down-regulated genes mostly remain unexplored. Actually, the transcriptional repression of some genes could also play an important role in cellular recovery from DNA damage. In the *E. coli* SOS response, diminished transcript levels have been clearly evident in some operons after UV irradiation (Courcelle et al., 2001). These operons control metabolism of carbon sources, lipid synthesis in membrane or

septum formation prior to cell division (Courcelle et al., 2001). The microarray assays in budding yeast have also shown that the transcription of some genes is repressed after treatment with DNA damaging agents (Gasch et al., 2001; Jelinsky et al., 2000; Jelinsky and Samson, 1999). The biological functions of these genes include nucleotide synthesis, assembly of ribosomal proteins and promotion of cell cycle progress. Collectively, these data suggest that cells may down-regulate some unnecessary metabolism and protein synthesis in response to DNA damage and slow down cell cycle progression to provide more time for damage repair through repressing the transcription of certain genes.

To date, the regulatory mechanism of transcriptional repression in response to DNA damage is not well understood. In *E. coli*, a large number of genes in wild type cells, but not in the *lexA* defective mutants, show reduced transcript levels after UV irradiation, suggesting that lexA is involved in inhibiting gene transcription in response to DNA damage (Courcelle et al., 2001). In mammalian cells, some transcriptional factors, such as p53 and E2F as discussed above, can actually function as both transcriptional activators and transcriptional inhibitors. p53 mediates transcriptional repression through interference with the basal transcriptional machinery (Ragimov et al., 1993; Subbaramaiah et al., 1999) and other transcriptional activators (Hoffman et al., 2002; Lee et al., 1999), or alternation of chromatin structure by recruiting proteins such as histone deacetylases (Mirza et al., 2002; Murphy et al., 1999). The mechanism for the repression activity of p53 is not clear. Given that phosphorylation of p53 at Ser386 appears to be necessary for transcriptional repression (Hall et al., 1996), the post-translational modification of p53 may contribute to its repression activity. It is also very possible that subtle differences within the p53-binding site itself may play pivotal roles in the repression activity of p53 (Ho and Benchimol, 2003).

# CHAPTER TWO

## MATERIALS AND METHODS

### 2.1. Bacterial culture and storage

The *E. coli* strain DH10B (Gibco/BRL, Grand Island, NY, USA) was used for bacterial transformation. Transformed cells were cultured in LB liquid or agar media (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 2% agar for plates) containing 100 μg/ml of ampicillin (Amp). For short-term storage (2 or 3 months), transformed cells were stored on LB + Amp plates. For long-term storage, transformed cells were grown overnight in 900 μl of LB + Amp and immediately placed in a –70$^{o}$C freezer after mixing with 100 μl of DMSO.

### 2.2. Preparation of competent cells

*E. coli* competent cells for electroporation were prepared as suggested in the BioRad *E. coli* Pulser (BioRad, Hercules, CA, USA) manual. One liter of culture was incubated at 37$^{o}$C until an $OD_{600nm}$ of 0.6 was reached. The culture was centrifuged at 2000 xg for 5 minutes in a Beckman GSA rotor and the pellet was resuspended in 500 ml of 10% ice-cold sterile glycerol. The centrifugation was repeated 4 times, with each pellet resuspended in a reduced volume; the last pellet was resuspended in 4 ml of ice-cold, sterile 10% glycerol. The cells were aliquoted into 1.5 ml eppendorf tubes to a volume of 25 μl, and were quickly placed in the –70$^{o}$C freezer for storage.

## 2.3. Bacterial transformation

All bacterial transformations in this study were carried out by the electroporation method. The DNA to be transformed was added to *E. coli* competent cells and the cell mixture was transferred to a pre-chilled electroporation cuvette (BioRad, Hercules, CA, USA). After a brief incubation on ice, the cells were exposed to a voltage of 1.8 kV (for cuvettes with 0.1 mm width) using the *E. coli* Pulser (BioRad). Four hundred microliters of SOC medium was added to the cuvette after electroporation. The cells were transferred to a 1.5 ml eppendorf tube, incubated at 37$^{o}$C for half an hour, spread on LB + Amp plates and incubated at 37$^{o}$C overnight.

## 2.4. Rapid preparation of plasmid DNA

Plasmid amplification and isolation was performed following the methods as described (Sambrook and Russell, 2001). Single colonies were inoculated into 1.5 ml LB + Amp liquid media and grown overnight at 37$^{o}$C. Cells were collected by centrifugation and the pellet was resuspended in 350 μl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0). After mixing with 20 μl of lysozyme (10 mg/ml; Sigma, St Louis, MI, USA), the mixture was quickly placed in a boiling water-bath for 40 seconds, followed by centrifugation for 10 minutes. The pellet was removed with a toothpick, and 8 μl of 5 M NaCl and 2 volumes of 95% ethanol were added to the sample to precipitate the DNA.

## 2.5. Agarose gel electrophoresis and DNA fragment isolation

For analysis of plasmid and genomic DNA, a 0.8% agarose gel was used in this study. Gels were run in 1 x TAE buffer (40 mM Tris-acetate, 2 mM $Na_2EDTA$) and stained with 0.5 μg/ml ethidium bromide (EtBr). DNA was visualized under UV light.

The method of DNA fragment isolation from agarose gels was adapted from Wang and Rossman (Wang and Rossman, 1994). After enzyme digestion, the sample was electrophoresed through a 0.6% agarose gel and stained with EtBr. The band of interest was identified using an UV-transilluminator and cut out of the gel. A 0.5 ml microcentrifuge tube was pierced at the bottom, and packed with chopped cheesecloth. The gel slice containing the DNA fragment was placed into the prepared tube, which was inserted into another 1.5 ml tube, left it in the $-70^oC$ freezer for 20 min and spun for 10 minutes at 16,000 xg. The flow through was extracted with an equal volume of phenol/chloroform (1:1) and then with chloroform. The DNA in the upper aqueous phase was precipitated by ethanol and resuspended in $H_2O$.

## 2.6. *S. cerevisiae* strains and cell culture

The *S. cerevisiae* strains used in this thesis are listed in Table 2-1. Yeast cells were cultured at $30^oC$ either in a rich YPD medium or in a synthetic dextrose (SD) medium. YPD is a standard, complex medium composed of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. SD medium is used for selective growth of yeast auxotrophs. It contains 0.67% yeast nitrogen base without amino acids, 2% glucose, and addition of any necessary auxotrophic supplements. The necessary auxotrophic supplements includes 30 mg/L L-isoleucine, 150 mg/L L-valine, 20 mg/L adenine hemisulfate salt, 20 mg/L arginine HCl, 20 mg/L L-histidine HCl monohydrate, 100

mg/L L-leucine, 30 mg/L lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 200 mg/L L-threonine, 20 mg/L L-tryptophan, 30 mg/L L-tyrosine, 20 mg/L L-uracil. Any of the above auxotrophic supplements can be omitted to provide a selection media for yeast transformation. The auxotrophic supplements were made as 100 × stocks and added into media prior to autoclaving. To make plates, 2% agar was added to either YPD or SD medium prior to autoclaving.

Yeast cells can be stored for up to four months on plates sealed with parafilm at 4°C. For long-term storage, yeast cells were grown in an appropriate liquid medium (rich or minimal media) at 30°C overnight. 0.7 ml of the culture was added into 0.3 ml of 50% sterile glycerol and then stored at -70°C.

## 2.7. Yeast transformation and targeted gene disruption

Yeast cells were transformed using a dimethyl sulfoxide (DMSO)-enhanced method as described (Hill et al., 1991). A 2 ml culture of yeast cells was grown overnight at 30°C in rich media (or appropriate minimal media), and subcultured into 3 ml of fresh media. When the yeast cells reached a mid-logarithmic phase of growth, they were pelleted by centrifugation. The yeast cells were washed in 400 µl LiOAc solution [0.1 M lithium acetate, 10 mM Tris-HCl (PH 8.0), 1 mM EDTA], and resuspended in 100 µl of the same solution. Five microliters of denatured carrier DNA (single stranded salmon sperm DNA 2 mg/ml) and 1-5 µl of transforming DNA were added and mixed well. After incubation at room temperature for 5 minutes, 280 µl of 50% of PEG4000 (50% polyethylene glycol 4000 in LiOAc solution) was added and mixed by inverting the tube 4-6 times. After the transformation mixture was incubated for 45

**Table 2-1. *Saccharomyces cerevisiae* strains**

| Strain | Genotype | Source |
|---|---|---|
| DBY747 | *MATa leu2-3,112 ura3-52 his3-Δ1 trp1-289* | D. Botetein |
| HK578-10A | *MATa ade2-1 can1 -100 his3-11,15 leu2-3112 trp-1-1 ura3-1* | H. Klein |
| HK578-10D | *MAT α ade2-1 can1-100 his3-11,15 leu2-3112 trp-1-1 ura3-1* | H. Klein |
| BY4741 | *MATa his3 leu2 met15 ura3* | Invitrogen |
| Y190 | *MATa gal4 gal80 his3 trp1 ade2-101 ura3 leu2 ura3::GAL1-lacZ lys2::GAL1-HIS3* | D. Gietz |
| SX46A | *MATa ade2(ochre), ura3-52, his3-532, trp1-289* | W. Siede |
| WXY9216 | DBY747 with *mag1Δ::hisG* | Laboratory stock |
| WXY9555 | DBY747 with *rad2Δ::TRP1* | Laboratory stock |
| WXY814 | DBY747 with *apn1Δ::HIS3 apn2Δ::LEU2* | Laboratory stock |
| WXY1164 | DBY747 with *rad51Δ::HIS3* | Laboratory stock |
| WXY9387 | DBY747 with *rad52Δ::LEU2* | Laboratory stock |
| WXY9376 | DBY747 with *rad6Δ::LEU2* | Laboratory stock |

| | | |
|---|---|---|
| WXY9326 | DBY747 with *rad18Δ::TRP1* | Laboratory stock |
| WXY9444 | DBY747 with *rad18Δ::LEU2* | Laboratory stock |
| WXY1248 | HK580-10D with *srs2*Δ*::LEU2* | Laboratory stock |
| WXY1297 | HK580-10D with *rad18*Δ*::LEU2 srs2*Δ*::HIS3* | Laboratory stock |
| BY4741 *siz1*Δ | BY4741 with *siz1*Δ*:: KanMX4* | Invitrogen |
| BY4741 *rad18*Δ | BY4741 with *rad18*Δ*:: KanMX4* | Invitrogen |
| WXY1190 | DBY747 with *rad24Δ::HIS3* | This study |
| WXY1198 | DBY747 with *sgs1Δ::LEU2* | This study |
| WXY1184 | DBY747 with *rad24Δ::HIS3 sgs1Δ::LEU2* | This study |
| WXY1185 | DBY747 with *rad18Δ::TRP1 sgs1Δ::LEU2* | This study |
| WXY1186 | DBY747 with *rad18Δ::TRP1 rad24Δ::HIS3* | This study |
| DSY1330 | SK1 *MATa ho::LYS2 lys2 ura3 trp1::hisG Leu2::hisG arg4-Bgl1 his4-X RAD53-6×MYC-KAN*$^{R}$ | D. Stuart |
| WXY1179 | DSY1330 with *rad18Δ::TRP1* | This study |
| WXY1180 | DSY1330 with *sgs1Δ::LEU2* | This study |

| | | |
|---|---|---|
| WXY1181 | DSY1330 with *rad24Δ::LEU2* | This study |
| WXY1182 | DSY1330 with *rad18Δ::TRP1 sgs1Δ::LEU2* | This study |
| WXY1183 | DSY1330 with *rad18Δ::TRP1 sgs1Δ::LEU2* | This study |
| WXY2760 | DBY747 with *rad17Δ::HIS3* | This study |
| WXY2307 | DBY747 with *rad18Δ::LEU2 rad17::HIS3* | This study |
| WXY2763 | DBY747 with *sgs1Δ::LEU2 rad17::HIS3* | This study |
| WXY2769 | DBY747 with *rad17-K197R* | This study |
| WXY2779 | DBY747 with *rad17-K197R sgs1Δ::LEU2* | This study |
| WXY1101 | DBY747 with *pol30Δ::HIS3* with YCpT-*POL30* | Laboratory stock |
| WXY1187 | DBY747 with *pol30Δ::HIS3* with YcpT-*pol30-K164R* | Laboratory stock |
| SX46A-Rad17-Myc | SX46A with *RAD17-13×Myc::KanMX4* | W. Siede |
| WXY1152 | BY4741 with *crt10Δ::KanMX* | Invitrogen |
| WXY1153 | BY4741 with *crt1Δ::KanMX* | Invitrogen |
| WXY1154 | BY4741 with *crt1Δ::KanMX crt10Δ::LEU2* | This study |
| WXY1155 | BY4741 with *dun1Δ::KanMX* | Invitrogen |

| | | |
|---|---|---|
| WXY1156 | BY4741 with *dun1Δ::KanMX crt10Δ::LEU2* | This study |
| WXY1157 | HK578-10A with *crt10Δ:: LEU2* | This study |
| WXY1158 | HK578-10D with *crt10Δ::LEU2* | This study |
| U952-3B | *MATa sml1Δ::HIS3* | R. Rothstein |
| U953-61A | *MATa mec1Δ::TRP1 sml1Δ::HIS3* | R. Rothstein |
| U960-5C | *MATa rad53Δ::HIS3 sml1-1* | R. Rothstein |
| WXY1159 | U952-3B with *crt10Δ::LEU2* | This study |

minutes at 30°C, 39 µl of DMSO was added, followed by a 5-minute heat shock in a 42°C waterbath. Yeast cells were then washed with sterile double distilled water ($ddH_2O$) and resuspended in 100 µl of $ddH_2O$. The resuspended cells were plated on the appropriate minimal media.

For targeted gene deletion, plasmid DNA was digested with restriction enzymes, precipitated by ethanol and resuspended in $ddH_2O$ prior to transformation. The transformants were verified by southern hybridization for target gene disruption.

## 2.8. Yeast genomic DNA isolation and Southern hybridization

To isolate genomic DNA for Southern hybridization, a protocol developed by Hoffman and Winston (Hoffman and Winston, 1987) was used. Yeast cells from liquid culture were collected by centrifugation and resuspended in 200 µl of extraction buffer [2% TritonX-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris HCl (pH 8.0)]. One hundred microliters of phenol, 100 µl of chloroform and 0.3 g of acid-washed glass beads were added to the cell mixture. The tube was then vortexed for 3 minutes (2 minutes for isolating plasmid DNA) at top speed. After a 5-minute centrifugation at top speed (16,000 xg), the top aqueous layer was transferred to a clean eppendorf tube. To precipitate DNA, 2 volumes of 95% ethanol were added. The tube was stored at -20°C for 30 minutes and centrifuged for 15 minutes at top speed. The DNA pellet was dried and resuspended in 200 µl of $ddH_2O$, and then treated with 5 µl of RNase A (10 mg/ml stock) at 37°C for 10 minutes. The DNA was precipitated by adding 2 volumes of 95% ethanol and resuspended in 50 µl of $ddH_2O$.

For Southern hybridization, genomic DNA was digested by appropriate restriction enzymes, and the DNA fragments were separated on a 0.8% agarose gel. The gel was

then treated in a solution of 0.25 M HCl for 10 minutes for depurination, in 0.4 M NaOH/0.6 M NaCl for 30 minutes for denaturation, and in 1.5 M NaCl/0.5 M Tris-HCl (pH 7.5) for 30 minutes for neutralization. The DNA was transferred from the gel to a nylon-based membrane (GeneScreen Plus, DuPont) in the presence of 10x SSC (3M NaCl, 0.3 M tri-sodium citrate, pH 7.0) overnight.

The following day, the membrane was placed into a hybridization bottle with 5 ml of pre-hybridization solution [2x SSC, 10% dextran sulphate, 5x Denhardt's solution (50 × stock: 10 g $Ficoll_{400}$, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin; $ddH_2O$ added to a total volume of 500 ml), 50% formamide, and 1% SDS] and incubated at 42°C in the hybridization oven for at least 2 hours. Before hybridization, 50 μl of boiled carrier DNA (2 mg/ml) and 60 μl of probe were added to the prehybridization solution. The membrane was then incubated with the probe overnight at 42°C. The membrane was washed twice for 5 minutes at room temperature in 2x SSC/0.1% SDS and washed twice for 30 minutes each at 65°C in 0.2x SSC/ 0.1% SDS. The membrane was then exposed to an X-ray film at -70°C with an intensifying screen.

The DNA probe was labelled with $^{32}$P-dCTP using the Random Primer Labelling kit (Gibco/BRL, Grand Island, NY, USA).

## 2.9. Plasmids and plasmid construction

Plasmid manipulation was performed using enzymes purchased from Invitrogen (Carlsbad, CA, USA) or New England Biolabs (Beverly, MA, USA) as recommended by the manufacturers.

Plasmids used for the detection of β-galactosidase (β-gal) activity, including YEp*MAG1-lacZ* and YEp*DDI1-lacZ* have been described previously (Liu and Xiao, 1997;

Xiao et al., 1993; Zhu and Xiao, 1998). Plasmids pZZ2 [YCp, *URA3*, *RNR3-lacZ*, (Allen et al., 1994)] and pZZ18 [YCp, *URA3*, *RNR2-lacZ*, (Allen et al., 1994)] were obtained from Dr. S. Elledge (Harvard Medical School, Boston, MA, USA) and utilized to determine *RNR3* or *RNR2* expression using a β-gal assay.

Sources of plasmids to make *rad18*Δ *::LEU2* (Xiao et al., 2000) and *rad17*Δ*::HIS3* strains (Zhu and Xiao, 1998) have been described. Plasmid pPWΔSGS1 (Watts, 2006) was obtained from Dr. I. Hickson (University of Oxford, UK) and the *sgs1*Δ*::LEU2* disruption cassette was released by *Nco*I-*Pst*I digestion.

To create the *rad24*Δ strain, *RAD24* genomic DNA was cloned into pTZ18R and a 1.64-kb *Eco*RV-*Nco*I fragment containing most of the *RAD24* open reading frame (ORF) including the start codon was deleted and replace by the 1.05-kb *HIS3* gene from YDp-H (Berben et al., 1991). The *rad24*Δ*::HIS3* cassette was released by *Bgl*II-*Hpa*I digestion.

To construct plasmid pGBT-Rad17 for the yeast two-hybrid assay, the *RAD17* ORF was amplified by PCR from genomic DNA with primers RAD17-1 (5'-CCCGGGGATCCGTATGCGAATCAACAGTGAGCTAG-3') and RAD17-2 (5'-GGCCGTCGACTTAAAAAAATATAGGAATATCC-3'). The PCR product was cloned into pGEM-T (Promega, Madison, WI, USA) to form pGEM-Rad17. The RAD17 ORF was released from pGEM-Rad17 by *Bam*HI and *Sal*I double digestion, and the isolated fragment was inserted into pGBT9 (Clontech, Mountain View, California, USA) to form pGBT-Rad17. To constructed pGBT-Mec3, the *MEC3* ORF was amplified by PCR with primers MEC3-2H-5' (5'-GGCCGGATCCATGAAATTAAAATTGA TAGTAAATG-3') and MEC3-2H-3' (5'- GGCCGTCGACTTACAAGCCC TTCGATCTTG-3'). The PCR product was cloned into pGEM-T to form pGEM-Mec3. pGEM-Mec3 was double

digested by *Bam*HI and *Sal*I to release the *MEC3* ORF, and the fragment was inserted into pGBT9-Bg (Fu and Xiao, 2003) to form pGBT-Mec3. The procedure to construct pGBT-Ddc1 was the same as that of pGBT-Mec3, except using primers DDC1-2H-5' (5'-GGCCGGATCCATGTCATTTAAGGCAACTATC-3') and DDC1-2H-3' (5'-GGCCGTCGACTTAGTCAAATATACCCCTTG-3') to amplify the *DDC1* ORF from genomic DNA by PCR.

To construct the Rad17-K197R::*URA3* integrating cassettes, a fragment containing Lys197Arg point mutation was amplified by PCR from pGEM-Rad17 by primers Rad17-K197R-5' (5'-CTGATATCTAGATCTCAGCTA-3') and RAD17-2 (5'-GGCCGTCGACTTAAAAAAATATAGGAATATCC-3'). The PCR product was double digested by *Eco*RV and *Sal*I, and then used to replace the original fragment in pGEM-Rad17 to form pGEM-Rad17-K197R. After validating the point mutation by sequencing, pGEM-Rad17-K197R was double digested by *Eco*RI and *Sal*I to release a 0.9-kb fragment. This fragment was inserted into pRS306 (Sikorski and Hieter, 1989) to produce YIpU-Rad17-K197R. For integration, YIpU-Rad17-K197R was digested by *Hin*dIII prior to yeast transformation. Meanwhile, the 0.9-kb fragment was inserted into pRS403 (Sikorski and Hieter, 1989) to form YIpH-Rad17-K197R. The Rad17-K197R::*HIS3* integrating cassettes was obtained by digesting YIpH-Rad17-K197R with *Eco*RV.

Plasmids YEpU-RAD6 and its C88A derivative were obtained from Dr. D. Gietz (University of Manitoba, Canada). Plasmid pGBT-RAD18 was made by cloning the *RAD18* ORF into pGBT9 and the C28S mutation was introduced by site-directed mutagenesis and confirmed by sequencing.

To construct YEp*DDI2*-lac*Z*, the promoter region of *DDI2* was amplified by PCR from genomic DNA by the primers YFL061w-1 (5'-GGAAAATCCAAGCTTTCAAG-3') and YFL061w-3 (5'-GCCGCGGCCGCCCTCATTGAAACTTACCT-3'). The PCR product was then cloned into YEp365R (Myers et al., 1986) to form YEp*DDI2*-lac*Z*. For the promoter deletion, the plasmid YEp*DDI2*-lac*Z* was digested by the corresponding restriction enzyme and religated. The UAS of *DDI2* promoter (-358 to -229) was amplified by PCR with the primers DDI2-UAS-1 (5'-ATCGCCCGGGCTTAACAGCAATGAAAATC-3') and DDI2-UAS-2 (5'-ATCGGCATGCGAAATGATAGTGTCCATGC-3'). The PCR product was inserted into pLG669Sm (Guarente and Hoar, 1984; Liu and Xiao, 1997) to create p*DDI2*-UAS-*CYC1-lacZ*.

To construct *crt10*Δ*::LEU2* disruption cassettes, the 2.8-kb *CRT10* ORF was amplified by PCR from genomic DNA with primers CRT10-1 (5'-CCGGAATTCATGCCCCCTCAGATTCCCAATG-3'), and CRT10-2 ( 5'-CGGGTC GACCTATTGTTGAGTTGTTCCATG-3'). The PCR product was cloned into pBluescript SK (Stratagene, La Jolla, CA, USA) to form pBS-CRT10. The resulting plasmid pBS-CRT10 was digested by *Hpa*I and *Msc*I to remove the fragment encoding aa60-871, and then ligated with a *Bgl*II linker to create plasmid pBS-crt10Δ. A *LEU2* marker was inserted into the plasmid to form pcrt10Δ::LEU2. For *CRT10* disruption, pcrt10Δ::LEU2 was digested by *Ava*I and *Nco*I prior to yeast transformation.

## 2.10. β-Galactosidase (β-gal) assay

The β-gal assay was performed as described previously (Fu and Xiao, 2006). A 0.5 ml fresh overnight culture of the yeast transformants was used to inoculate 2.5 ml of

minimal medium without Leu, His or Ura, depending on the selectable marker of the *lacZ* fusion plasmids. Two sets of cells were allowed to grow for another 2 hours. Methyl methanesulfonate (MMS) was added to one set of cells at the desired concentration, and both sets were incubated at 30$^{o}$C for 4 hours. One ml of cells was used to determine cell titer based on optical density at 600 nm. The remaining 2 ml of cells were collected and permeabilized in 1 ml of Buffer Z (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO4$, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0), by adding 50 µl of 0.1% SDS, 50 µl of chloroform and votexing at top speed for 10 sec. The reaction was started by adding 200 µl of 4 mg/ml orthonitrophenyl-β-D-galactosideae (ONPG) and incubating at 30$^{o}$C for 20 minutes. The reaction was stopped by adding 500 µl of 1 M $Na_2CO_3$, and 1 ml of supernatant was transferred into a cuvet to determine the OD at 420nm. The β-gal activity was determined using the following equation: β-gal activity = (1000 × $OD_{420nm}$) / [Reaction time (minute) × Culture volume (ml) × $OD_{600nm}$], and is expressed in Miller units. The β-gal assay was performed with several independent yeast transformants from the same transformation to avoid internal inconsistency. Results from various transformants/treatments presented for comparison (e.g., treated *vs* untreated, full length promoter *vs* its derivates) were always from the same experiment to avoid inter-experimental variation. All the results presented are the average of at least three independent experiments.

### 2.11. RNA isolation, reverse transcription and hybridization to microarray

Yeast cells were grown to early log phase and treated with or without 0.1% MMS for 48 minutes. Total RNA was isolated by the RNeasy Midi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The positive control Arabidopsis RNA

was generated through *in vitro* transcription of *Sac*I digested pARAB (UHN Microarray Centre, Toronto, ON, Canada). *In vitro* transcription was performed by the MEGAscript$^{TM}$ T7 kit (Ambion, Huntingdon, Cambridgeshire, UK), following the manufacturer's protocol. Ten microgram of total RNA template and 4 ng control Arabidopsis RNA were reverse transcribed to generate Cy3 or Cy5 labelled cDNA using AncT primer ($T_{20}$ VN) and Superscript II RT enzyme (Invitrogen) for 2 hours of incubation at 42$^{o}$C. Four microliters of 50 mM EDTA (pH 8.0) and 2 µl of 10 N NaOH were added to stop the reverse transcription, and a further 20 minutes of incubation at 65$^{o}$C was performed to hydrolyse the remaining RNA. Fluorescence-labelled cDNA was purified by Microcon columns (Millipore, Billerica, MA, USA). The yeast 6.4K oligonucleotide Arrays (UHN Microarray Centre) were used in this study. The hybridization solution was prepared by mixing DIG Easy Hyb (Roche, Basel, Switzerland), sonicated calf thymus DNA (Sigma) and yeast tRNA (Invitrogen) with a 20:1:1 ratio. The fluorescence-labelled cDNA was combined with the hybridization solution, and the mixture was loaded on the oligonucleotide arrays. The arrays were incubated at 37$^{o}$C overnight for hybridization under sealed high moisture condition to prevent drying up. The following day, the arrays were washed three times for 15 minutes each in the 1 × SSC, 0.1% SDS solution at 50$^{o}$C, followed by rinsing with 1 × SSC at room temperature to remove traces of SDS. After spinning dry at 500 rpm for 5 minutes, the arrays were ready for scanning. Two independent microarray assays were carried out by generating Cy5 labelled cDNA from MMS-treated cells and Cy3 labelled cDNA from untreated cells. In order to avoid biased incorporation of Cy5 and Cy3, the reverse color experiments were performed. In this case, Cy5 was incorporated into first-strand cDNA which was synthesized by using the RNA from untreated cells, and the fluorescent dye

Cy3 was incorporated into cDNA by using the RNA from cells treating with MMS as the templates. The average values were taken for the further Microarray data analysis.

## 2.12. Microarray data analysis

Array images were acquired using a GenePix 4000B microarray scanner (Axon Instruments, Union city, CA, USA). The image from each microarray chip was analyzed by the GenePix Pro 6.0 software (Axon Instruments, Union city, CA, USA) to create a GenePix Array List (GAL) file. Microarray data was analyzed by Acuity 3.1 software (Axon Instruments). Transcripts defined as regulated by Rad6/Rad18 met the criteria of: (1) after treatment with MMS, gene induction in the wild type strain was at least double the untreated levels; (2) the expression levels in the *rad6*Δ mutant and *rad18*Δ mutants were nearly the same (based on a Self-Organizing Map analysis with Acuity 3.1); (3) the expression levels were significantly decreased in the *rad6*Δ and *rad18*Δ mutants compared to wild type cells (1.5 fold or greater, one way anova test $P < 0.01$).

## 2.13. Northern hybridization and real-time PCR

Northern hybridization was performed as described previously (Zhu and Xiao, 1998). Briefly, RNA was isolated by a glass-bead method (Carlson and Botstein, 1982). Yeast cells were cultured overnight at 30$^{o}$C in an appropriate liquid medium, subcultured in 4 ml medium next day and incubated for 4 more hours. Cells were collected by centrifuging at 3000*g* for 4 minutes at room temperature, and washed twice with 0.1 % diethylpyrocarbonate (DEPC)-treated water. Three hundred and fifty microliters of RNA lysis buffer (0.5 M NaCl, 10 mM EDTA, 1% SDS, 0.2M Tris-HCl pH7.6), 0.4 g of acid-washed glass beads and 350 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were

added. The tubes were vortexed at top speed for 2.5 minutes, and centrifuged at 16,000 xg for 4 minutes. The aqueous phase was transferred into a new microcentrifuge tube. Two volumes of ethanol were added, and the tubes were immediately centrifuged at 16,000 xg for 4 minutes. The RNA pellet was washed with 200 µl 70% ethanol, dried briefly under vacuum, and dissolved in 40 µl DEPC water. The RNA sample was separated by gel electrophoresis, blotted on a *GeneScreen Plus* membrane (NEN) and hybridized with $\alpha$-$^{32}$P-labelled DNA probe. After an overnight hybridization, the membrane was washed and exposed to a phosphorimager screen. The mRNA band intensity was measured with a Molecular Imager FX (Bio-Rad) supported by the Quality One 4.2.1 software. The source and preparation of *ACT1* probes has been described previously (Zhu and Xiao, 1998). The 0.7-kb *DDI2* ORF was amplified by PCR using DDI2-2 (5'-GCCGAATTCATGTCACAGTACGGATTT-3') and DDI2-3 (5'-GCCGCGGCCGCCCTCATTGAAACCTACCT-3') as primers.

For real-time PCR, yeast cells with or without treatment were harvested from early log phase culture ($2 \times 10^7$ cells/ml). Total RNA was prepared using an RNeasy midi Kit (Qiagen). The extracted RNA was treated with Ambion's DNA-free™ Kit to remove contaminating DNA. The treated RNA was used as the template to perform reverse transcription with the Thermoscript™ RT-PCR system (Invitrogen). Real-time PCR was carried out and analyzed with iCycler® Thermal Cycler (Biorad). The primers used in real-time PCR were listed in Table 2-2. The relative transcript level of each treatment was determined by a method and formula as follow: ratio= $(E_{target})^{\Delta Ct \text{ target (Control-treated)}}$ / $(E_{reference})^{\Delta Ct \text{ reference (Control-treated)}}$ (E represents the efficiency for the primers; $\Delta$Ct represents the difference in the Ct values, which is the point at which the fluorescence crosses the threshold. *ACT1* was used in this study as the reference genes) (Pfaffl, 2001).

**Table 2-2. The primers used in Real-time PCR**

| Primer | Sequence |
|---|---|
| RNR3-1 | 5’-GCCTCCGCTGCTATTCAA-3’ |
| RNR3-2 | 5’-CAGATGCCGCCTTTTGTT-3’ |
| MAG1-RT1 | 5’-GCGGTGCATTTCCTGATTA-3’ |
| MAG1-RT2 | 5’-TCGCGAGCCTCCAAAGTAT-3’ |
| ACTIN-RT1 | 5’-TGGCCGGTAGAGATTTGACTGACT-3’ |
| ACTIN-RT2 | 5’-AGAAGCCAAGATAGAACCACCAAT-3’ |
| TUP1-1 | 5'-CCACCACGTCGACGGATAACAATA-3' |
| TUP1-2 | 5'-CTCGGAATCCCAAACT CTCACAGC-3' |
| SSN6-1 | 5'-GCCCAAGCTCCCCAACC-3' |
| SSN6-2 | 5'-CTGTGCGCCAATTACTGAAGG-3' |
| CRT1-1 | 5'-GGTCGCCCGTTAAACAGAGTA-3' |
| CRT1-2 | 5’-CGTGGGCGATATAGAGTTAGAGT-3' |

## 2.14. Cell killing by DNA damaging agents

A gradient plate assay was one of the methods used to measure MMS sensitivity. Thirty millilitres of molten YPD agar were mixed with an appropriate concentration of MMS to form the bottom layer. The gradient was created by pouring the medium into a tiled square petri dish. After brief solidification for one hour, the petri dish was returned to a flat position and 30 ml of the same molten agar without MMS was poured to form the top layer. A 0.1 ml sample was taken from an overnight culture, mixed with 400 µl sterile water and 0.5 ml of molten YPD agar, and then immediately imprinted onto freshly made gradient plates using a microscope slide. Gradient plates were incubated at $30^{o}$C for 2 days before photographs were taken.

For the HU sensitivity analysis, log phase yeast cells were diluted to 1 x $10^7$ cells/ml, and 10-fold serial dilutions were made. Aliquots of 10 µl of diluted cells were spotted on the appropriate plates, and the plates were incubated at 30°C for 3 days.

## 2.15. The yeast two-hybrid assay (Y2H) to assess protein interactions

Yeast strain Y190 was received from Dr. D. Gietz and used for Y2H. Y190 was transformed simultaneously with a combination of $Gal4_{BD}$ (e.g., pGBT9, pGBT-Rad17, pGBT-Ddc1, pGBT-Mec3) and $Gal4_{AT}$ (e.g., pGAD424, pGAD-Rad18, and pGAD-Rad6) plasmids. For each combination, the co-transformed colonies were initially selected on SD-Trp-Leu plates. At least four independent colonies from each combination were further spotted on SD-Leu-Trp-His plates or plates SD-Leu-Trp-His plus different concentration of 3- aminotriazole (3-AT) plates to test the activation of $P_{GAL}$-*HIS3* gene. Plates were incubated at 30°C for 48 hours.

## 2.16. Yeast protein extraction and western blotting

Yeast protein crude extraction was performed as previously described (Pellicioli et al., 1999). Ten millilitres of yeast cell culture (0.5-1× $10^7$ cells/ml) were spun down to collect cells. The cell pellet was washed with 1 ml of 20% trichloroacetic aicd (TCA) solution. After centrifugation, the cells were resuspended in 200 µl of 20% TCA. Glass beads were added to the meniscus, and the tube was vortexed for 2-4 minutes. An additional 400µl of 5% TCA were added to the meniscus and the aqueous extract was transferred to a new tube. After spinning at 16,000 xg for 10 minutes, the supernatant was discarded, and the pellet was mixed with 100 µl of protein sample buffer (150 mM Tris PH 6.8, 6% SDS, 30% glycerol, 0.3% bromophenol blue and 15% β-mercaptoethanol) and 50 µl of 1 M Tris-base. The sample was incubated at 100$^o$C for 5 minutes and further spun in a microfuge at 800 xg for 10 minutes. The supernatant was transferred to a new tube, and the crude extract could be preserved at -20$^o$C.

Protein samples were boiled, run on a 10% SDS-PAGE gel, and then transferred to a PVDF membrane (polyvinylidene difluoride, Bio-Rad). Membranes were treated in a blocking solution (PBS, 5% non-fat milk) at room temperature for 1 hour. Anti- Myc antibodies (Upstate, Lake placid, NY, USA) were diluted 1:5000 in 20 ml PBST [PBS, 0.05% Tween (v/v), 1% non-fat milk] and the PBST solution was incubated with membranes at 4$^o$C overnight. The second antibody, anti-rabbit IgG conjugated with HRP, was used at a 1:10000 dilution. The Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Science, Boston, MA, USA) was utilized for detecting, and the membrane was then exposed to an X-ray film.

For Rad17 immunoprecipitation analysis, one liter of log-phase cultures of SX46A or SX46A-RAD17-Myc harbouring different plasmid combinations were treated

with or without 0.05% MMS for 90 minutes. The cell pellet was collected by centrifugation at 2000 xg at 4°C for 10 min, and washed with ice-cold PBS once and ice-cold PBS + 0.1% NP40 twice. After resuspending the cell pellet in 3 ml of PBS + 0.1% NP40, 5ml of glass beads, as well as protease inhibitor cocktails (Sigma), Phenylmethylsulfonyl Fluoride (PMSF, Sigma) and N-Ethylmaleimide (NEM, Sigma), were added to the meniscus. The yeast cells were disrupted by votexing at top speed on ice for 4 minutes. The supernatant was transferred to new falcon tubes, and was sonicated on ice for 10 seconds three times. The yeast lysate was then spun at 17,000 xg for 30 minutes. The supernatant (crude protein extraction) was carefully transferred to a new tube for further experiments.

Protein G–sepharose beads (Sigma) were used for immunoprecipitation. Approximately 200 µl of beads were washed by 1.6 ml of PBST (PBS + 0.5g/L Tween-20, $Ca^{2+}$/$Mg^{2+}$ -free) 3-4 times in a screw-capped Eppendorf tube. The beads were re-suspended in 1.6 ml of PBST containing 5 µl anti-Ubiquitin polyclonal antibody (Upstate), 50 mg BSA and 0.01% thimersol. The tubes were set horizontally on a gentle rocking platform to keep the beads in motion. The rocking was continued overnight at 4°C. The following day, the pre-absorbed beads were spun down at 2,000 xg for 2 minutes. After removing the supernatant, 1 ml of yeast crude protein extract was added to the Eppendorf tube. The mixture was then carefully transferred to a 15 ml Falcon tube, and 5 ml of yeast crude protein extract were added. The Falcon tubes were placed horizontally on a gently rocking platform overnight on ice. The following day, the beads were spun down at 2000 xg for 2 minutes. The supernatant was removed, and pre-chilled PBST was added to wash the beads. The washing was repeated at least 8 times. To elute the protein from the beads, 100 µl of protein loading buffer with fresh 10 mM

Dithiothreitol (DTT) was added and heated to 95°C for 5 minutes. After boiling, iodoacetimide was added to Eppendorf tubes to a concentration of 25 mM from a fresh 0.5 M stock to inhibit disulfide bonds from reforming. After gentle vortexing, the tubes were spun down at 16,000 xg for 1 minute. The supernatant was carefully collected without disturbing the beads and loaded onto a protein gel.

## 2.17. Screening of yeast gene deletion library

The yeast haploid gene deletion library was created by the *Saccharomyces* Genome Deletion Project Consortium and purchased from Research Genetics (Invitrogen, Carlsbad, CA). The deletion mutants were replica plated on to YPD and YPD + 80 mM HU. Plates were incubated at 30°C for 3 days before evaluation.

## 2.18. Yeast tetrad analysis

For tetrad analysis, parental haploid strains U953-61A and WX1158 were streaked on YPD plates. Two days later, two strains were cross-streaked in an X-formation and mixed at the centre of the X on SD-Trp-His-Leu plates which would support the growth of the diploid, but neither of the haploid cells could grow. The plates were incubated at 30°C for 2-3 days to obtain individual diploid colonies, which were inoculated into 2 ml of YPD medium and cells were allowed to grow overnight at 30°C with constant agitation and aeration. The following day, cells were washed 2 times with sterile dd$H_2O$, re-suspended in 5 ml of sporulation media (0.5% potassium acetate, 0.5 × auxotrophic nutrients), and incubated at room temperature for 3 to 7 days with agitation and aeration.

Sporulation was checked by visual inspection with a light microscope after 3 days. If there were tetrads present, 10 µl of media was transferred to a sterile Eppendorf tube and 10 µl of NEE-154 glusulase (Dupont Company, Wilmington, DE, USA) was added. After incubation at room temperature for 10 minutes, 20 µl of ice-cold dd$H_2O$ was added and the tube was put on ice immediately. The tetrads were dissected on YPD plates with a Singer MSM micromanipulator (Singer Instrument Co. Somerset, England). The YPD plates were incubated at 30$^{o}$C for 3 days to allow the growth of tetrads. After 3 days of incubation, the genotypes of tetrads were identified by replica plating to YPD and SD medium containing appropriate combinations of amino acids.

# CHAPTER THREE

## Rad6-Rad18 Mediates a Eukaryotic SOS Response by Ubiquitinating the 9-1-1 Checkpoint Clamp

### 3.1. Introduction

DNA integrity is challenged by the damaging effect of numerous chemicals and physical agents, and these damages probably lead to mutations within chromosomal DNA, which may cause cancer or premature aging. Organisms respond to these insults to DNA by activating complex DNA damage response pathways. These pathways regulate DNA damage responses through processes such as cell cycle arrest, DNA repair, transcriptional regulation and apoptosis (Zhou and Elledge, 2000). When bacteria are subjected to DNA damage, a reaction known as the SOS response co-ordinately regulates DNA repair, transcription and cell division (Little and Mount, 1982; Michel, 2005). Substantial progress made in the past several years suggests that the checkpoint pathway, which was originally thought to control primarily cell cycle progression, regulates multiple responses including transcription, DNA repair and apoptosis in eukaryotes (Zhou and Elledge, 2000). Thus, for purposes of clarity, DNA damage response has been suggested to refer to the entire pathway and use "the checkpoint branch" to refer components specially involved in controlling cell-cycle progression (Zhou and Elledge, 2000).

The DNA damage response pathway is a signal transduction cascade consisting of sensors, transducers and effectors. Proteins that initially sense damaged DNA to trigger signal transduction are currently unknown. In principle, a sensor protein should have the ability to interact with damaged DNA. In mammalian cells, poly (ADP-ribose)

polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) have been proposed as DNA damage sensors due to their ability to bind and be activated by DNA strand breaks. However, genetic evidence indicates that these proteins are not activators of the global DNA damage response (Jimenez et al., 1999; Wang et al., 1995; Zhou and Elledge, 2000). Human and *Schizosaccaromyces pombe* Rad9-Rad1-Hus1 (9-1-1) complexes and their homologous Ddc1-Rad17-Mec3 complex in *S. cerevisiae*, which is related in structure to PCNA, share some of the properties expected for sensors. This doughnut-like heteromer complex can be loaded onto damaged DNA just as PCNA is loaded onto primed DNA (Komatsu et al., 2000; O'Connell et al., 2000; Volkmer and Karnitz, 1999). Furthermore, the complex is required for the DNA damage checkpoint branch and plays a role in DNA repair (Parrilla-Castellar et al., 2004). Nevertheless, genetic evidence is still missing to prove that it is indeed a sensor and to demonstrate its role in transcriptional regulation following DNA damage. DNA repair proteins have also been suggested as candidate sensors since they can recognize aberrant DNA structures after DNA damage. For example, it was reported that the NER machinery which processes UV photoproducts is required to activate a *RAD9*-dependent checkpoint response in budding yeast (Giannattasio et al., 2004; Siede et al., 1994). Additionally, the MRE11-RAD50-NBS1 (*Sc*Xrs2) complex, which functions in the non-homologous end joining repair pathway, is involved in inducing autophosphorylation of ATM kinase in mammalian cells (Carson et al., 2003; Uziel et al., 2003).

If DNA repair proteins function as a sensor to detect DNA damage and induce the global DNA damage response, these proteins should play a role in the regulation of gene expression in response to DNA damage. To test this hypothesis, we examined a panel of isogenic yeast gene deletion strains for their ability to support *MAG1-lacZ* and *DDI1-*

*lacZ* reporter gene expression in the presence and absence of DNA damage induced by MMS, a chemical carcinogen specifically inducing replication blocks. *MAG1* and *DDI1* are two divergently transcribed genes found in the budding yeast *Saccharomyces cerevisiae* (Chen et al., 1990; Liu and Xiao, 1997; Xiao et al., 1993), and are involved in DNA repair (Chen et al., 1989) and regulation of exocytosis (Lustgarten and Gerst, 1999), respectively. Both genes are DNA damage inducible and contain common as well as unique *cis*-acting regulatory elements (Liu and Xiao, 1997; Xiao et al., 1993). Deletion of *MAG1* (representing base excision repair), *RAD2* (representing nucleotide excision repair), *RAD52* (representing homologous recombination) had little effect on the induction of both reporter genes, suggesting that they are not essential for transcriptional regulation. In contrast, deletion of *RAD6* or *RAD18* (representing postreplication repair, PRR) significantly reduced the expression of both reporter genes. Further analysis indicates that this novel regulatory function of *RAD6* and *RAD18* is independent of its PRR activity since deletion of downstream PRR genes *MMS2*, *REV3* or double deletion of *MMS2* and *REV3* had no effect on the induction of *MAG1* (Zhu, 2003).

In this study, by a microarray analysis, we further demonstrated that deletion of *RAD6* and *RAD18* reduces the DNA damage-induced transcription of up to 379 genes. These genes include those involved in DNA repair, control of replication and transcription, regulation of the cell cycle and cell metabolism. It was found that Rad18 shares the pathway with Rad24, rather than Sgs1, to regulate the phosphorylation of Rad53 in checkpoint pathway. Furthermore, the Ddc1-Rad17-Mec3 complex appears to play a role in the Rad6/Rad18 regulatory pathway.

## 3.2. Results

### 3.2.1. *RAD6* and *RAD18* are involved in the DNA damage induction

Previous research in our laboratory demonstrated that deletion of *RAD6* or *RAD18* (representing postreplication repair, PRR) significantly reduced the expression of both *MAG1-lacZ* and *DDI1-lacZ* reporter genes, but deletion of *RAD2* (representing nucleotide excision repair, NAR) and *RAD52* (representing homologous recombination, HR) has little effect on the induction of both reporter genes (Zhu, 2003). In order to further confirm these observation, the expression of *MAG1-lacZ* and *DDI1-lacZ* reporter genes were measured in *apn1Δ apn2Δ* double mutants (representing base excision repair, BER), *rad51Δ* mutants (representing HR) and *pms1Δ* mutants (representing mismatch repair, MMR). Unlike deletion of *RAD6* or *RAD18*, deletion of these genes did not significantly reduce the expression of reporter genes (Figure 3-1), suggesting that they are not required for the signal transduction leading to transcriptional regulation. Thus, it is likely that only the post-replication repair pathway genes *RAD6* and *RAD18* are involved in the DNA damage-induced gene regulation.

### 3.2.2. Lack of DNA damage induction in *rad6* or *rad18* strains is not due to the severely enhanced sensitivity to DNA damaging agents

The *rad6Δ* and *rad18Δ* mutants display severely enhanced sensitivity to DNA damaging agents (Fabre et al., 1989; Swietlinska et al., 1976). It remains a possibility that lack of DNA damage induction in *rad6Δ* or *rad18Δ* is due to their extreme sensitivity to DNA damaging agents. However, there are several lines of evidence that argue against

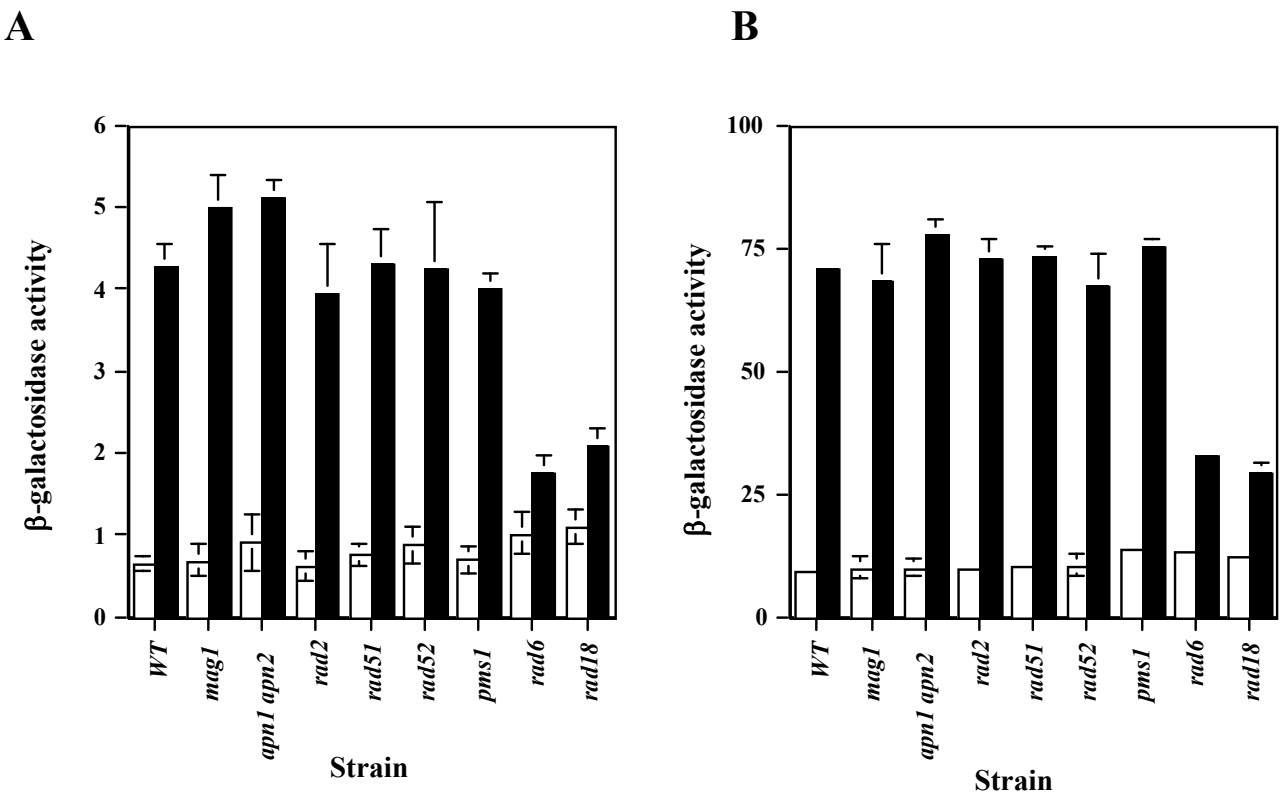


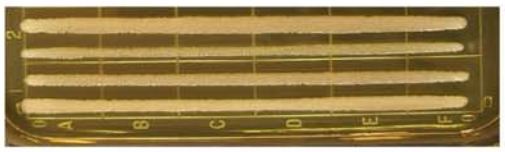

**Figure 3-1. MMS-induced expression of *MAG1* and *DDI1* is compromised in *rad6* and *rad18* cells.** *MAG1-lacZ* (**A**) and *DDI1-lacZ* (**B**) expression in a series of DNA repair mutants defective in base excision repair (*mag1* and *apn1 apn2*), nucleotide excision repair (*rad2*), homologous recombination repair (*rad51* and *rad52*), mismatch repair (*pms1*) or PRR (*rad6* and *rad18*) with (solid bars) or without (open bars) 0.05% MMS treatment for 4 hours. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

this notion. Firstly, it was reported that deletion of *SRS2* can significantly alleviate DNA damage sensitivity of *rad6* and *rad18* mutants (Schiestl et al., 1990). Further deletion of *SRS2* in a *rad18Δ* strain alleviated its MMS sensitivity, but did not restore the DNA damage inducibility of *MAG1-lacZ* and *DDI1-lacZ* (Figure 3-2). Secondly, inactivation of both error-free and error-prone branches of the *RAD6* PRR pathway also results in severe MMS sensitivity (Broomfield et al., 2001), but does not affect *MAG1* or *DDI1* induction (Zhu, 2003). Finally, other DNA repair mutant strains, such as *rad52*Δ and *apn1Δ apn2Δ* double mutants (Hanna et al., 2004), are also extremely sensitive to killing by MMS, but they did not affect the *MAG1* and *DDI1* inducibility (Figure 3-1).

### 3.2.3. Deletion of *RAD6* and *RAD18* globally decreases the DNA damage induction in budding yeast.

In order to determine the role of Rad6/Rad18 in DNA damage induction at the total genome level, a DNA microarray analysis was performed. After treating cells with 0.1% MMS for 48 minutes, the transcript levels of 751 genes were increased by 2-fold or higher in wild type cells, which is comparable to other reports using Gene Chip (Jelinsky and Samson, 1999) or cDNA from different sources (Gasch et al., 2001). Among these genes, 379 genes showed similar transcript levels in *rad6*Δ and *rad18*Δ mutants based on the Self-Organizing Map analysis with Acuity 3.1, and a decreased transcriptional level by 1.5-fold or greater with a significance of $P < 0.01$ (one way anova test) compared to wild type cells (Figure 3-3). The percentage of genes affected by *rad6* or *rad18* mutations appears to increase among those with higher level induction (Table 3-1). Of the 379 genes, 39% of them code for proteins whose functions have not been

A



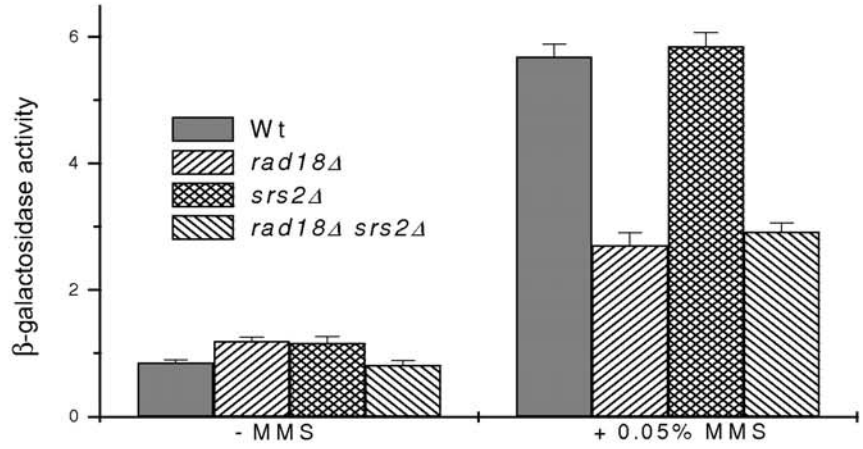
*rad18Δ srs2Δ*
*srs2Δ*
*rad18Δ*
Wt

YPD

*rad18Δ srs2Δ*
*srs2Δ*
*rad18Δ*
Wt

YPD + 0.0075%
MMS

B




C

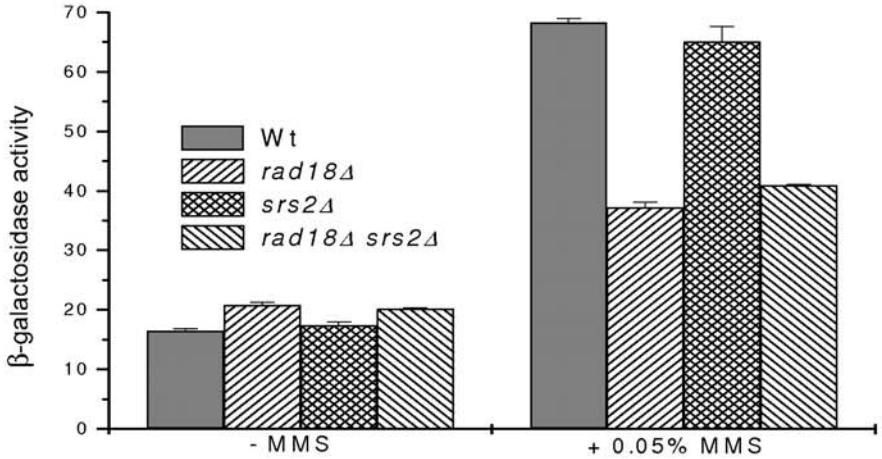


**Figure 3-2. Deletion of *SRS2* suppresses the extreme sensitivity of *rad18* but does not restore the induction of *MAG1* and *DDI1*.** (**A**) A gradient plate assay to measure relative MMS sensitivity of wild type, *rad18Δ*, *srs2Δ* and *rad18Δ srs2Δ* mutants. Arrows point to higher MMS concentration. The plates were incubated for two days at 30$^{o}$C. *MAG1-lacZ*(**B**) and *DDI1-lacZ*(**C**) expression in wild type (W303) and its isogenic *rad18*Δ, *srs2*Δ, *rad18*Δ *srs2*Δ derivatives. Log-phase cells were either treated with 0.05% MMS for 4 hours or without treatment prior to β-gal assays. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

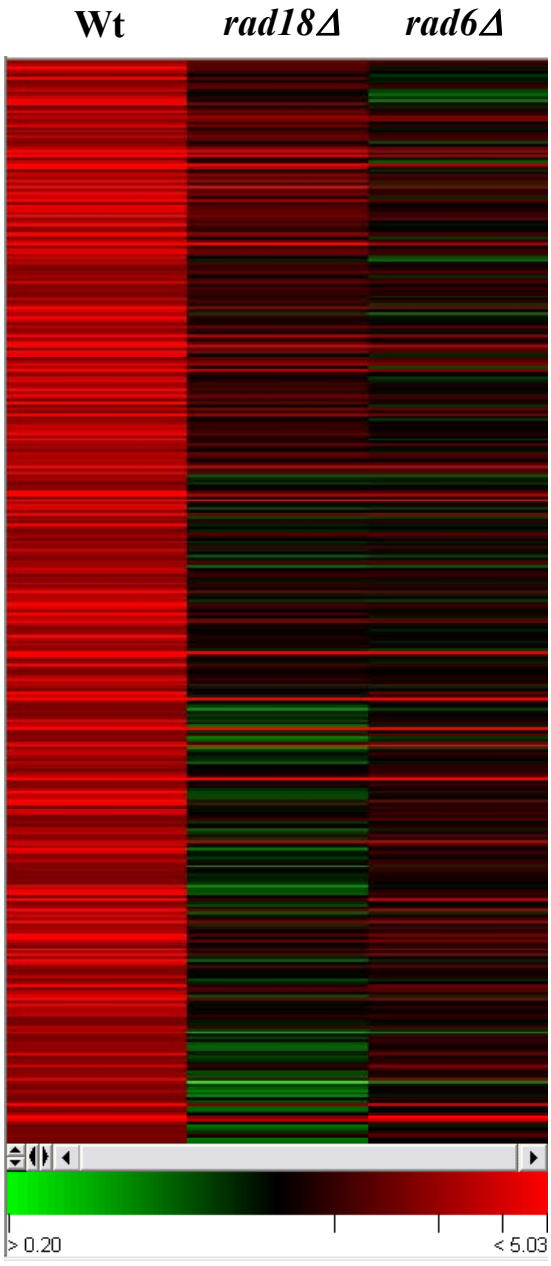



**Figure 3-3. Gene expression data from the microarray analysis**. The dendrogram is based on 379 genes that passed the filtering criteria. The list of genes shown in red/green ratio (indicated in the bottom) represents the ratio of expression level of MMS-treated *vs.* untreated cells. A detailed expression profile is given in Appendix A.

**Table 3-1. Effects of *rad6* and *rad18* on DNA damage-inducible gene expression**

| **Induced by MMS** | **≥ 2 fold** | **≥ 3 fold** | **≥ 4 fold** |
|---|---|---|---|
| # of genes induced | 505 | 127 | 122 |
| # of genes affected by *rad6/rad18* | 213 | 74 | 92 |
| % affected | 42% | 58% | 75% |

characterized according to the *Saccharomyces* Genome Database (SGD, www.yeastgenome.org, Stanford University). The remaining 61% of genes encode proteins involved in numerous biological functions as assessed by SGD, including DNA repair, control of replication and transcription, regulation of the cell cycle and cell metabolism (Figure 3-4). The complete list of 379 genes is detailed in the Appendix A.

### 3.2.4. Microarray data validation

To ensure the accuracy of our microarray data, a validation process was conducted by comparing the genes known to be affected by *rad6* or *rad18* mutation with the data collected from the microarray.

We have demonstrated that the induction of *MAG1* and *DDI1* decreases in *rad6*Δ or *rad18*Δ mutants through both β-gal assays and northern blot analyses (Zhu, 2003). Based on our microarray data, the induction level of *MAG1* and *DDI1* in wild type cells was 2.2- and 5.6-fold, respectively, whereas the induction of *MAG1* and *DDI1* was decreased by 1.3- and 2.6-fold, respectively, in the *rad6*Δ mutant, and by 1.4- and 2.2 -fold in the corresponding *rad18*Δ mutant.

The transcript level of *RNR3* is induced over 50-fold when yeast cells are treated with 0.01% MMS, while the induction level decreases when treated with higher concentrations of MMS (Jia et al., 2002). In the microarray experiment, yeast cells were treated with 0.1% MMS for 48 minutes, which induces *RNR3* by only 3.3-fold in wild type cells. In *rad6*Δ and *rad18*Δ mutants, the induction level was decreased by 1.2- and 0.9-fold, respectively. Similarly, the transcript level of *PHR1* increased 3.1-fold in wild type cells, but only reached 1.5- and 1.6-fold, respectively in *rad6*Δ and *rad18*Δ mutants. Although the quantitative change for each gene was not exactly the same between

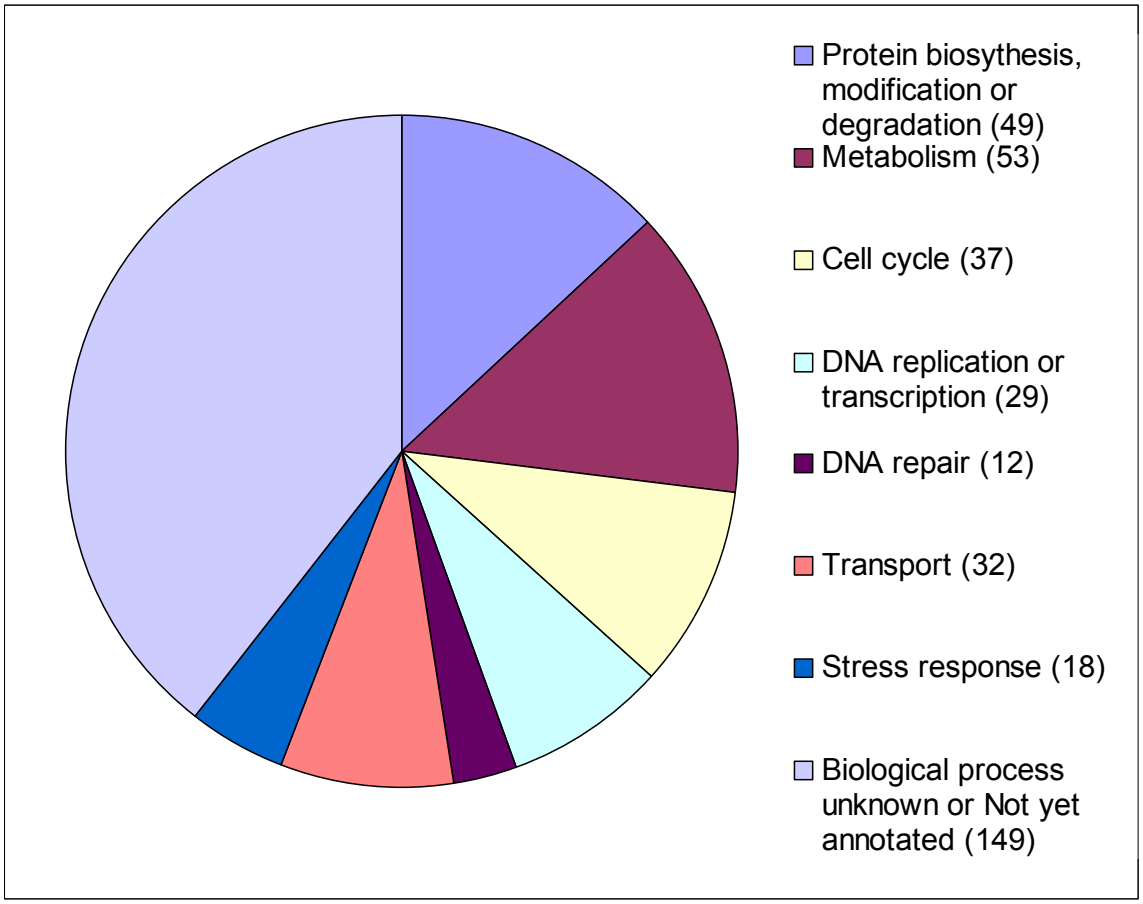



**Figure 3-4. Distribution of *RAD6-RAD18* regulated genes based on their functional annotation.** The 379 yeast genes whose DNA damage induction decreased in both *rad6* and *rad18* mutants were analyzed based on SGD and presented as a percentage of the total number of the affected genes.

northern blot, β-gal assay and microarray analyses, the general trend of being down-regulated in *rad6*Δ and *rad18*Δ mutants was consistent among these experimental results.

Under our microarray experimental conditions, the expression of *YFL061w* and *YNL335w* were induced by MMS over 100-fold, which is consistent with a previous report (Jelinsky and Samson, 1999). Interestingly, these two ORFs are identical and are located in a large region of gene duplication, including their promoter regions (SGD, www.yeastgenome.org, Stanford University). We designated the ORFs *YFL061w* and *YNL335w* as *DDI2* and *DDI3* for DNA damage inducible gene 2 and 3, respectively. The induction level of *DDI2* was found to be decreased in both *rad6*Δ and *rad18*Δ mutants by about 20-fold in the microarray analysis (Appendix A). To further validate the microarray result, we performed northern blot analysis; the results (Figure 3-5) correlated very well with the microarray data.

### 3.2.5. Domains required for DNA damage induction in Rad6

Rad6 functions in PRR as well as sporulation, meiotic recombination (Prakash et al., 1993) and telomere silencing (Huang and Elledge, 1997), In contrast, deletion of *RAD18* only has a pronounced defect on PRR. Furthermore, *RAD6* has been previously reported to participate in transcriptional regulation through ubiquitination of histone H2B (Kao et al., 2004). Construction of specific mutations has already identified regions in Rad6 required for different cellular functions. In order to determine whether these regions are required for DNA damage induction, different plasmids harboring specific *RAD6* mutations were introduced into the *rad6Δ* mutant together with the *RNR3-lacZ* plasmid and the β-gal assay was performed. The Cys88 in Rad6 abolishes its ubiquitin-conjugating activity, and the *rad6-C88A* mutations confer a defect in PRR, mutagenesis,

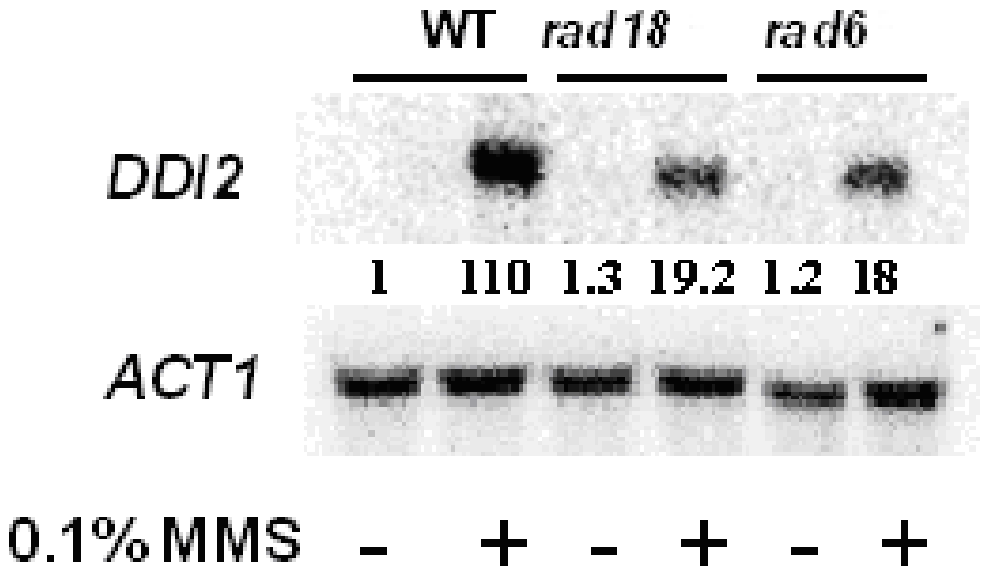



**Figure 3-5. Reduced *DDI2* induction in *rad6* and *rad18* mutants as measured by northern hybridization.** Wild type and mutant cells were treated with 0.1% MMS for 48 minutes prior to RNA isolation and northern hybridization. Each lane contains 15 μg of total RNA. The blot was stripped and hybridized with the *ACT1* probe. The *DDI2* transcript level in each sample was normalized with reference to that of *ACT1*, and expressed relative to the value of untreated wild-type sample in the same blot. Strains used in the above studies were DBY747 and its isogenic *rad6*Δ or *rad18*Δ derivatives.

and sporulation (Sung et al., 1990). After MMS treatment, the induction of *RNR3-lacZ* in *rad6-C88A* mutant was dramatically decreased compared with that in wild type cells (Figure 3-6). Deleting the highly acidic 23-residue carboxy-terminal tail domain of Rad6 (*rad6-C*) results in the loss of histone-polyubiquitinating activity (Robzyk et al., 2000; Sung et al., 1988). However, deleting this region had no effect on DNA damage induction of *RNR3-lacZ* (Figure 3-6). Removal of the 9-residue N-terminus of Rad6 (rad6-*N*) abolishes its N-end rule protein degradation function (Sung et al., 1991; Watkins et al., 1993), and this mutation decreased the DNA damage induction of *RNR3-lacZ* equivalent to that of the *RAD6* deletion mutant (Figure 3-6). Taken together, these observations demonstrate that Cys-88 and the N-terminal 9 amino acid residues in Rad6, but not its carboxyl-terminal polyacidic tail, are required for its DNA damage induction function.

### 3.2.6. PCNA modifications are not involved in the DNA damage induction of *MAG1* and *DDI1*

*RAD6* and *RAD18* encode a ubiquitin conjugating enzyme (Ubc or E2) and a ubiquitin ligase (Ubl or E3), respectively, that form a stable complex (Bailly et al., 1997). The only activity known to date of this complex is to monoubiquitinate a *POL30* product proliferation cell nuclear antigen (PCNA) at the Lys164 residue and to initiate the PRR pathway (Hoege et al., 2002), although the Rad6 E2 activity is involved in a number of other pathways independent of the Rad18 E3 activity (Broomfield et al., 2001). It remains possible that PCNA monoubiquitination, but not the subsequent translesion synthesis (TLS), is required for DNA damage induction. To test this hypothesis, we

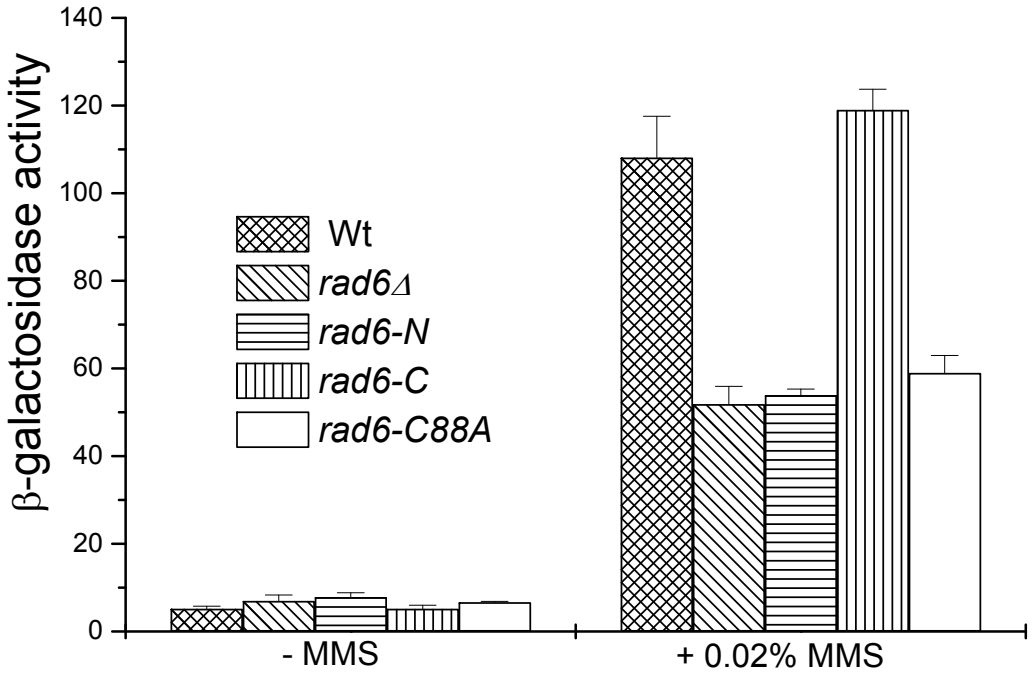


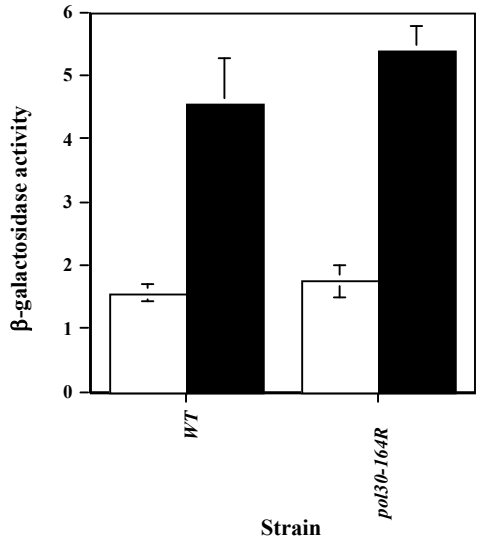

**Figure 3-6. Rad6 domains required for its DNA damage induction function.** *RNR3-lacZ* expression was analyzed in *rad6Δ* mutants harboring different plasmids that contain intact Rad6 or Rad6 deletion mutants. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

measured *MAG1-lacZ* activity in a *pol30-K164R* mutant. The *pol30-K164R* mutation overrides the Rad6-Rad18 activity in PRR but has no effect on *MAG1-lacZ* expression (Figure 3-7A). Hence, PCNA monoubiquitination is not required for DNA damage induction. PCNA can also be modified by a small ubiquitin-like modifier (SUMO) at K164 and K127 and a SUMO ligase Siz1 is required for both modifications (Stelter and Ulrich, 2003). We measured *MAG1-lacZ* activity in the *siz1* mutant and observed no alteration in the *MAG1-lacZ* induction (Figure 3-7B). The above results allow us to conclude that covalent modifications of PCNA by Ub or SUMO and the subsequent PRR activity is dispensable for *MAG1* and *DDI1* induction.

### 3.2.7. Rad18 is involved in the phosphorylation of Rad53

The yeast cell cycle checkpoints, including replication checkpoints and DNA damage checkpoints, are required for the DNA damage induction of a number of genes (Jang et al., 1999; Kiser and Weinert, 1996; Navas et al., 1995; Zhou and Elledge, 1993; Zhu and Xiao, 1998), and the checkpoint response has been suggested to function like a eukaryotic SOS response (Aboussekhra et al., 1996; Zhou and Elledge, 1993). Indeed we have previously shown that inactivation of key checkpoint genes such as *POL2* (Polε), *MEC1*, *RAD53* or *DUN1* severely affects *MAG1* induction (Zhu and Xiao, 1998; Zhu and Xiao, 2001). Others have reported that the above checkpoint genes are also required for the damage induction of *RNR* genes (Navas et al., 1995; Zhou and Elledge, 1993) and some other genes (Gasch et al., 2001; Kiser and Weinert, 1996). However, the extent of checkpoint gene involvement appears to vary with different target genes. For example, damage checkpoint genes *RAD9*, *RAD17* and *RAD24* are reported to affect

**A**




**B**

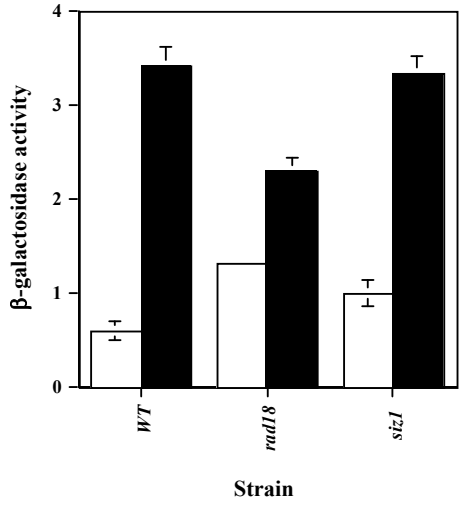



**Figure 3-7. PCNA covalent modifications are not required for *MAG1* induction**. (**A**) The PCNA K164R substitution does not affect *MAG1-lacZ* induction. (**B**) The *SIZ1* deletion that abolishes PCNA sumoylation does not affect *MAG1-lacZ* induction. Log-phase cells were either treated with 0.05% MMS for 4 hours (solid bars) or without (open bars) treatment prior to β-gal assays. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

*RNR* gene induction (Aboussekhra et al., 1996), but has little effect on *MAG1* (Zhu and Xiao, 1998), whereas *PHR1* induction requires Rad53 but not Dun1 (Jang et al., 1999). The Mec1-Rad53-Dun1 pathway forms a central kinase cascade responsible for the transcriptional regulation branch of the checkpoint response, whereas Rad53 phosphorylation has been utilized as a sensitive and reliable assay for checkpoint activation. Using a strain containing a Myc-tagged Rad53, we found that deletion of *RAD18* reduced Rad53 phosphorylation to a small extent compared to the reported inactivation of other *RAD53* upstream genes such as *MEC1*. It has been reported that *RAD24* and *SGS1* form two alternative branches required for Rad53 phosphorylation; simultaneous inactivation of both genes results in a synergistic reduction of Rad53 phosphorylation (Frei and Gasser, 2000; Myung and Kolodner, 2002). We reasoned that Rad6-Rad18 may function through one of the above two branches. Corresponding *rad18 rad24* and *rad18 sgs1* double mutants were created and compared with their respective single mutants with respect to the effect on damage-induced Rad53 phosphorylation. As seen in Figure 3-8, among all the mutants tested, only the *rad18 sgs1* double mutant displayed a dramatic reduction in Rad53 phosphorylation. This result suggests that Rad6-Rad18 functions in the Rad24 checkpoint pathway parallel to the Sgs1 pathway to induce Rad53 phosphorylation. This hypothesis predicts that the effect of *rad18* and *rad24* mutations on the induction of DNA damage inducible genes is epistatic, whereas that of *rad18* or *rad24* is additive to the *sgs1* mutation. Indeed, the *lacZ* reporter gene assays for both *MAG1* (Figure 3-9A) and *RNR3* (Figure3-9B) confirmed the above prediction. In particular, the *RNR3-lacZ* activity is reduced by four-fold in the *rad18 sgs1* and *rad24 sgs1* double mutants, compared to about a 30% reduction in the *rad18* and *rad24* single mutant or the *rad18 rad24* double mutant. Finally, to determine

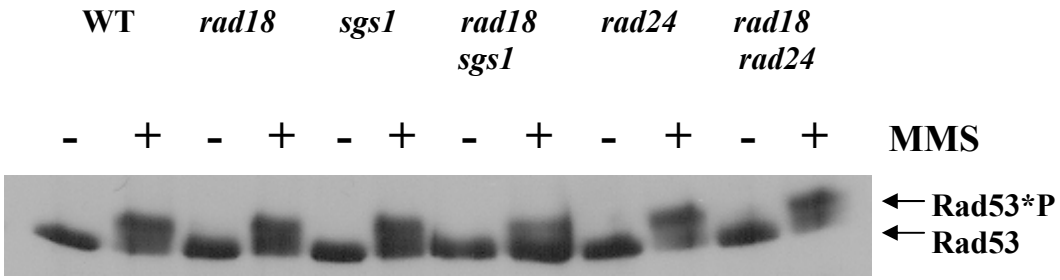



**Figure 3-8. Rad18 acts in the same pathway as Rad24 in the phosphorylation of Rad53 in response to DNA damage.** Log-phase cultures of isogenic strains were treated with (+) or without (-) 0.1% MMS for 1 hour and the Rad53 phosphorylation status was monitored by western blotting using an anti-Myc antibody against Myc-tagged Rad53. The shifted phosphorylated Rad53 (Rad53*P) bands disappeared after treating the protein samples with lambda protein phosphatase (data not shown).

whether the *lacZ* reporter assay faithfully represents the expression of endogenous genes, we measured the endogenous *MAG1* transcript level by a quantitative real-time PCR method. It was found (Figure 3-9C) that in response to MMS treatment, the *MAG1* transcript level is reduced by more than two-fold in *rad18* and *rad24* single mutants. Nevertheless, the *rad18 rad24* double mutant has the same *MAG1* transcript level as the corresponding single mutants. In contrast, the *rad18 sgs1* and *rad24 sgs1* double mutants displayed *MAG1* induction levels barely above those without MMS treatment (Figure 3-9C). In summary, with respect to DNA damage induction of *MAG1*, the effect of *rad18 sgs1* and *rad24 sgs1* double mutations is comparable to that of the *rad53*, *mec1* or *dun1* single mutations (Zhu and Xiao, 1998; Zhu and Xiao, 2001), indicating that *RAD6/RAD18-RAD24* and *SGS1* constitute two major signal transduction pathways. We suspect that in the case of *RNR3*, a third branch exists that is independent of the above two pathways but depends on the central *MEC1-RAD53-DUN1* signal transduction pathway.

### 3.2.8. Rad18 genetically and physically interacts with Rad17

In budding yeast, Rad24 and Rad17 checkpoint proteins are involved in early responses to DNA damage in a signal transduction pathway leading to cell cycle arrest. Rad24 interacts with the four small subunits of replication factor C (RFC) to form the RFC-Rad24 complex (Shimomura et al., 1998). Rad17 forms a complex with Mec3 and Ddc1 (Rad17/Mec3/Ddc1), and this complex shows structural similarities with the replication clamp PCNA (Kondo et al., 1999; Majka and Burgers, 2003). The RFC-Rad24 clamp loader loads the Rad17/Mec3/Ddc1 clamp around damaged DNA sites in an ATP-dependent process (Majka and Burgers, 2003). In *S. pombe* and mammalian cells,

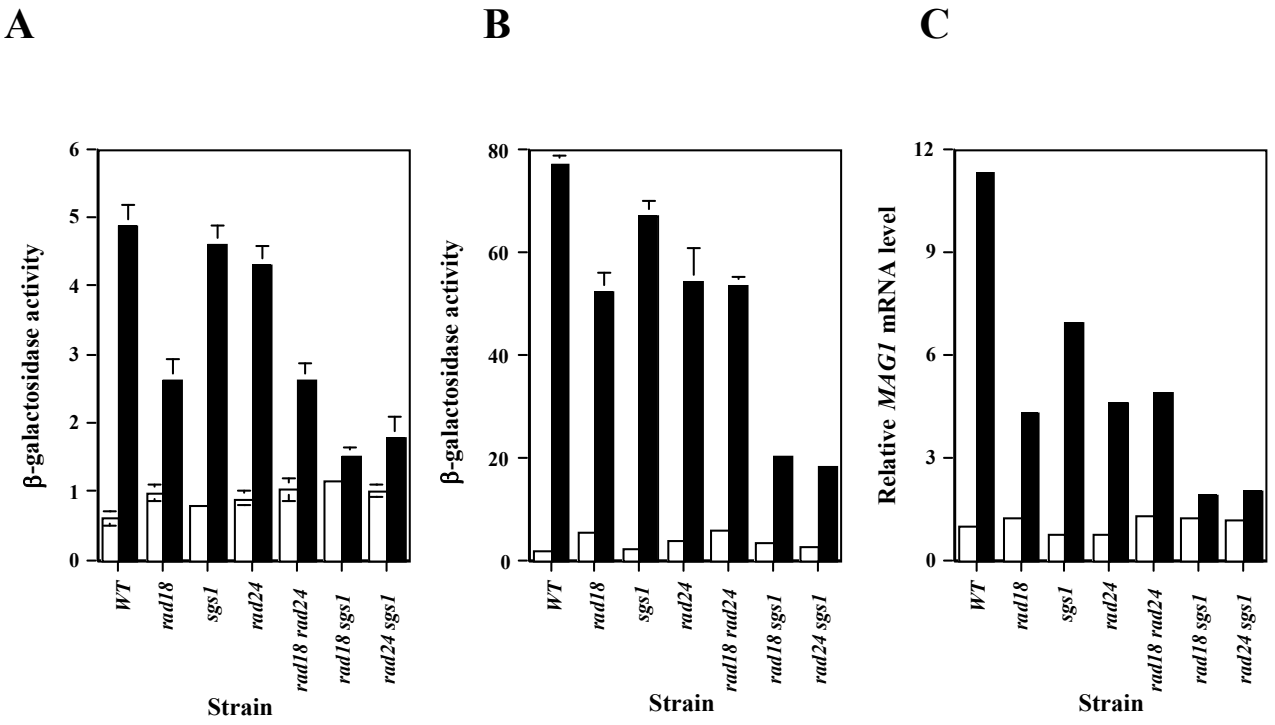



**Figure 3-9. *MAG1* and *RNR3* expression in response to MMS treatments in *rad18*, *rad24*, *sgs1* single or the corresponding double mutants.** The expression of *MAG1-lacZ* (**A**) and *RNR3-lacZ* (**B**) were measured by β-gal assay. Cells were untreated (open bars) or treated with 0.05% (**A**) or 0.02% (**B**) MMS for 4 hours (solid bars) prior to β-gal assays. Results are in Miller units and represent the average of at least three experiments with standard deviations. (**C**) Quantitative measurement of the *MAG1* transcript level in various mutants by real-time PCR. Cells were treated with 0.05% MMS for 30 min (solid bars) or remained untreated (open bars) prior to RNA isolation and the real-time PCR reaction was performed as described.

the homolog of Rad17, Mec3 and Ddc1 are Rad1, Hus1 and Rad9 respectively, and this complex is also termed the 9-1-1 clamp. Since the Rad18/Rad6 complex and Rad24 belong to the same signal transduction pathway, it is possible that the Rad18/Rad6 complex functions with the PCNA-like complex Rad17/Mec3/Ddc1 (9-1-1 clamp) to regulate gene induction. This hypothesis is supported by the results from β-gal assays of *MAG1-lacZ* in *rad17Δ*, *rad17Δ rad18Δ* and *rad17Δ sgs1Δ* mutants. Similar to *RAD24*, deletion of *RAD17* in *rad18Δ* mutants did not further decrease the DNA damage induction of *MAG1-lacZ*, while the MMS-induced expression of *MAG1-lacZ* was dramatically decreased in the *rad17Δ sgs1Δ* double mutant, compared to the expression in either the *rad17Δ* mutant or the *sgs1Δ* single mutant (Figure 3-10). This indicates that Rad18/Rad6 and 9-1-1 complexes belong to the same pathway that regulates gene induction. Interestingly, in a yeast two-hybrid assay, Rad18 showed weak interaction with Rad17 and the level of interaction is comparable to that of Pol30 with Rad18, but significantly less than that of Rad6 with Rad18 (Figure 3-11). In contrast, neither Ddc1 nor Mec3 showed an interaction with Rad18 (Figure 3-11). Furthermore, the lack of interaction of Mec3 or Ddc1 with Rad18 was not due to lack of expression or proper folding, since they were able to interact with Rad17 in the same yeast two-hybrid assay (Figure 3-11). Thus, it is hypothesized that Rda18/Rad6 regulates DNA damage induction through ubiquitination of Rad17.

### 3.2.9. *RAD6/RAD18*- and DNA Damage-dependent Mono-ubiquitination of Rad17

To examine whether Rad17 is ubiquitinated in a Rad6/Rad18-dependent manner, we used a chromosomally Myc-tagged Rad17 strain and monitored Rad17-Myc modifications by western blot analysis (Figure 3-12A). Western blot analysis revealed a

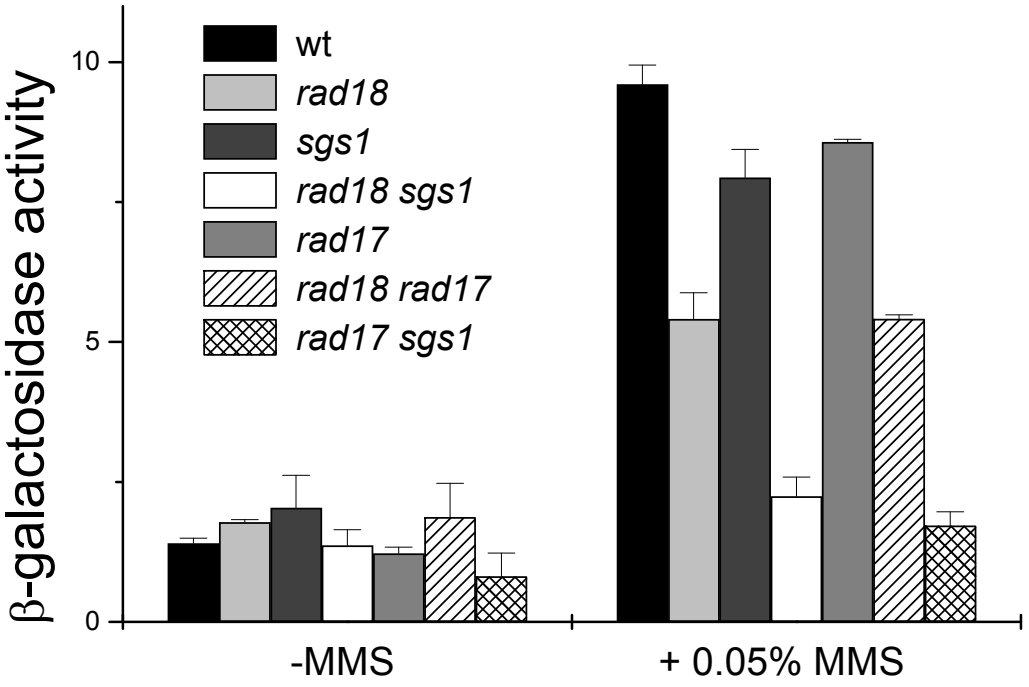



**Figure 3-10.** ***MAG1-lacZ*** **expression in response to MMS treatment in** ***rad18*****,** ***rad17*****,** ***sgs1*** **single or the corresponding double mutants.** Results are in Miller units and represent the average of at least three experiments with standard deviations.

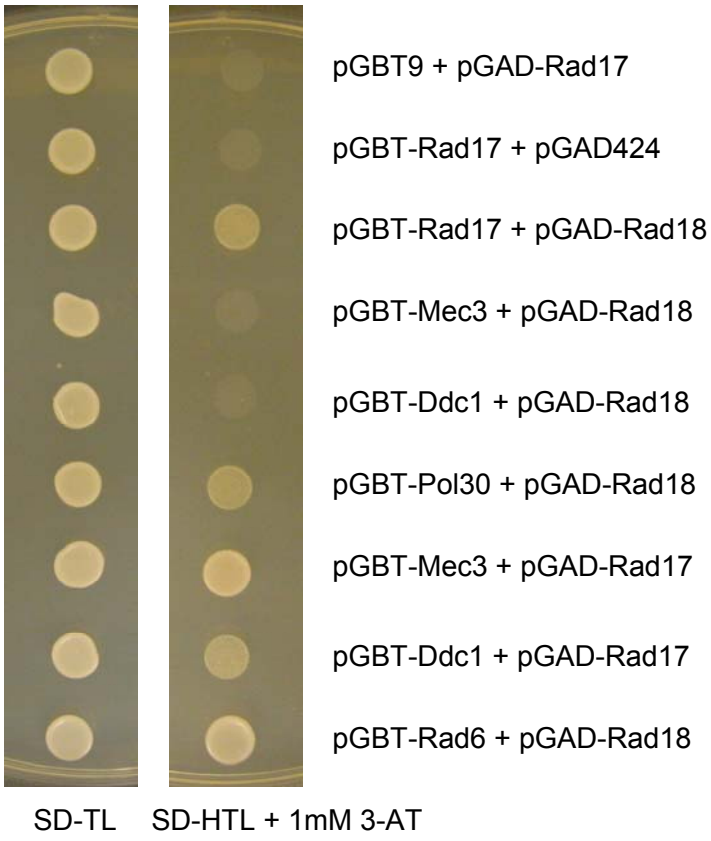



**Figure 3-11. Rad18 interacts with Rad17, but not with Ddc1 and Mec3.** Yeast strain Y190 was co-transformed with various combination of pGBT9, pGBT-Rad17, pGBT-Ddc1, pGBT-Mec3, pGBT-Pol30, pGBT-Rad6 and pGAD-Rad18, pGAD-Rad17, pGAD424. The co-transformants were dropped on SD-TL and SD-HTL with 1 mM 3-AT plates. All plates were incubated at 30°C for 3 days.

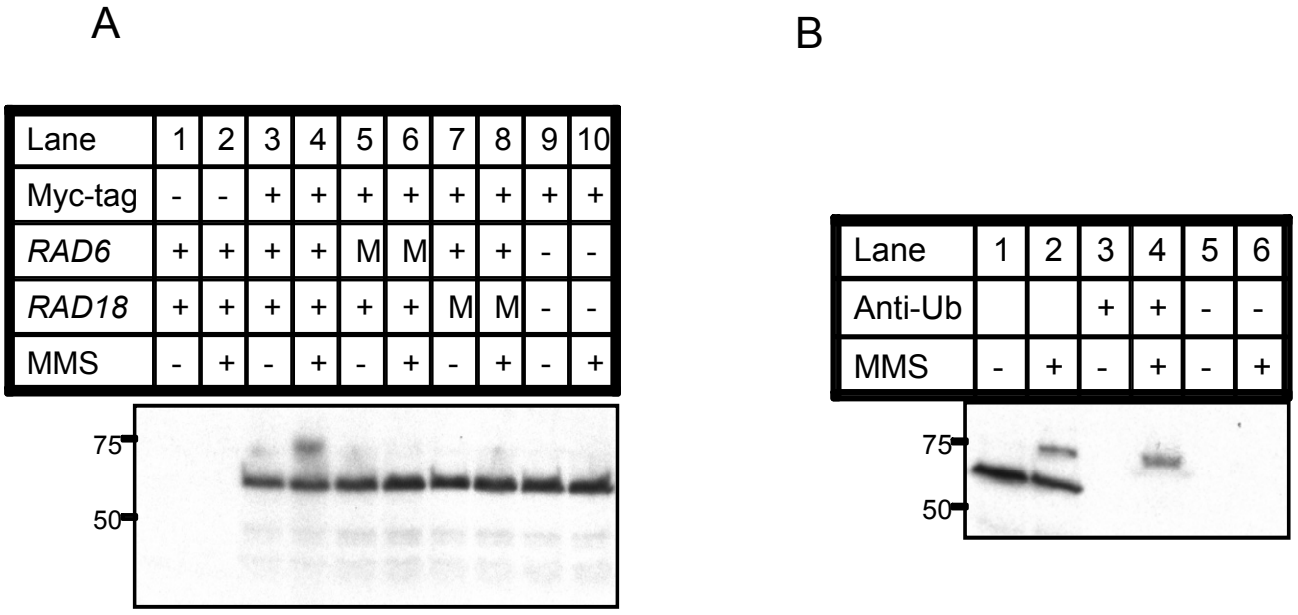



**Figure 3-12. Rad17 Is the Substrate for Rad6-Rad18 Mono-ubiquitination after DNA Damage.** (**A**) Western blot analysis of whole-cell lysates with an anti-Myc antibody against Rad17-Myc. Lanes 1 and 2 were lysates from SX46A and the remaining lanes were from SX46A-R17-Myc. Cells harbor both YEp-RAD6 and pGBT-RAD18 (+), or their mutant version YEp-RAD6-C88A or pGBT-RAD18-C28S (M), or the corresponding vectors (-) as indicated on the top panel and grew under the same conditions. (**B**) Western blot analysis of anti-Ub IP products with the anti-Myc antibody. Lanes 1 and 2 contain whole-cell lysates as controls. Lanes 3 and 4 contain IP products with the anti-Ub antibody, whereas lanes 5 and 6 contain IP products under the same experimental conditions but without the anti-Ub antibody. All yeast cells harbor YEp-RAD6 and pGBT-RAD18 plasmids. MMS treatment was at 0.05% for 90 minutes.

band consistent in size with the expected Rad17-Myc (63.5 kDa) but failed to detect its modification under both MMS-treated and untreated conditions (see lanes 9 and 10). We thought that this was probably due to the limit of either the detection sensitivity or the endogenous level of Rad6 and/or Rad18. Indeed, a modified Rad17-Myc band was reproducibly detected in cells overexpressing both *RAD6* and *RAD18* and treated with MMS (lane 4). This band is deemed specific for the Rad17-Myc modification since the isogenic cells without the Rad17-Myc-tag did not reveal both modified and unmodified bands (lanes 1 and 2). This modification also appears to depend on MMS treatment, although we cannot rule out the possibility of background modification of Rad17-Myc in untreated cells (lane 3).

The migration of the modified band agrees well with the expected size of mono-ubiquitinated Rad17-Myc (72 kDa). Furthermore, in cells harboring plasmids containing a single *rad6-C88A* active-site mutation (lane 6) or a *rad18-C28S* RING finger mutation (lane 8), this modification was not observed, implying that it is indeed the mono-ubiquitination of Rad17-Myc. To further confirm the above assumption, we performed immunoprecipitation (IP) by first using an anti-Ub antibody for affinity precipitation and then probing with the anti-Myc antibody. As shown in Figure 3-12, the anti-Ub antibody precipitated a protein that can be detected by the anti-Myc antibody and comigrates with the modified Rad17-Myc band from the whole-cell extract (cf. lanes 2 and 4). In contrast, unmodified Rad17-Myc was not detected after IP (lane 4), confirming the high degree of anti-Ub IP specificity. Cell lysates collected without the anti-Ub antibody during Co-IP (lane 6) or with anti-Ub but without MMS treatment (lane 3) did not display the same modified Rad17-Myc band. Furthermore, in all experiments, we did not observe additional modified Rad17-Myc bands indicative of poly-ubiquitination. Taken together,

we conclude that Rad17 is mono-ubiquitinated in a Rad6-Rad18 and DNA damage-dependent manner.

### 3.2.10. The *rad17-K197R* mutant is defective in DNA damage induction

The amino acid sequence alignment showed that the region around Lys197 in Rad17 shares significant homology with the region around the Lys164 in Pol30 (Figure 3-13 A). Since the Rad6/Rad18 complex ubiquitinates Pol30 at Lys164 residue (Hoege et al., 2002), it is possible that the Rad6-Rad18 complex could also ubiquitinate Rad17 at the Lys197 residue to initiate DNA damage induction. To test this hypothesis, we created a mutant strain in which the single lysine 197 residue present in Rad17 was changed to Arginine (*rad17-K197R*). In *rad17* mutants lacking the putative ubiquitination site K197 (*rad17-K197R*), DNA damage induction of *MAG1-lacZ* decreased to the same level as the *RAD17* deletion mutants (Figure 3-13B). Meanwhile, the effect of *rad17-K197R sgs1Δ* on *MAG1-lacZ* expression is also comparable to the *rad17Δ sgs1Δ* double mutation. All these data imply that ubiquitin modification of the Lys197 residue in Rad17 might be crucial for DNA damage induction.

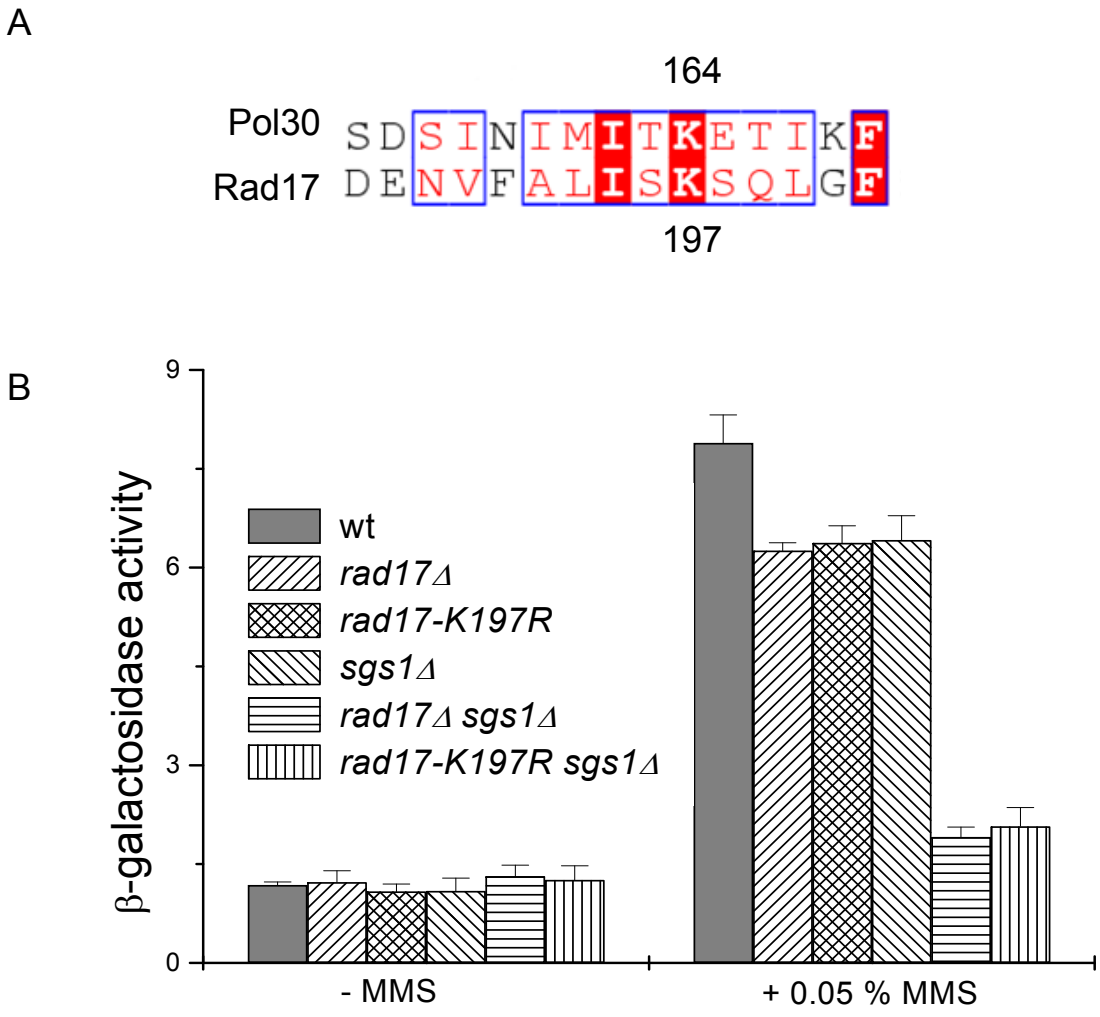



**Figure 3-13. The *rad17-K197R* mutant is defective in DNA damage induction.** (**A**) Sequence alignment of Pol30 (residues 155 to 168) and Rad17 (residues 188 to 202). Pol30-K164 and rad17-K197 are marked. (**B**) The expression of *MAG1-lacZ* was measured in *rad17Δ*, *sgs1Δ*, *rad17-K197R*, *rad17Δ sgs1Δ*, and *rad17-K197R sgs1Δ* mutants by a β-gal assay. Cells were untreated or treated with 0.05% MMS for 4 hours prior to the β-gal assays. Results are in Miller units and represent the average of at least three experiments with standard deviations.

## 3.3. Discussion

### 3.3.1. New Roles of *RAD6* and *RAD18* in DNA Damage-induced Gene Regulation

We report that two PRR genes, *RAD6* and *RAD18*, are involved in the regulation of a large number of DNA damage-inducible genes. *RAD6* has been previously reported to participate in transcriptional regulation. For example, *RAD6* is required for the transcriptional repression at telomeres and the *HM* loci (Huang and Elledge, 1997), and for the ArgR/Mcm1 repression of *ARG1* in the arginine biosynthesis pathway (Turner et al., 2002). The detailed mechanism of the above transcriptional repression has not yet been elucidated. One possibility is that Rad6 mediates the degradation of certain transcriptional regulators through the ubiquitin proteosome pathway. At least one transcriptional factor, Gcn4, is regulated by the Rad6 ubiquitination pathway (Kornitzer et al., 1994). Alternatively, Rad6's involvement in the ubiquitination of histone subunit H2B (Robzyk et al., 2000) may lead to an open chromatin structure or mark chromatin for recognition by regulatory proteins, which in turn activate the transcription of certain target genes (Kao et al., 2004). In any event, the above two processes involve only Rad6 but not Rad18. Indeed, *RAD18* has not been linked to the transcriptional regulation of any genes prior to this study.

To the best of our knowledge, this is the first report that *RAD6* and *RAD18* function in transcriptional regulation in response to DNA damage. Since the only known activity of the Rad6-Rad18 complex is in PRR, it strongly suggests that PRR is responsible for the observed gene regulation. To our surprise, among all the PRR mutations examined, only *rad6* and *rad18* affected the expression of damage-inducible genes, indicating that the *RAD6-RAD18* function in gene regulation is independent of

PCNA ubiquitination. This led to the identification of the PCNA-like clamp 9-1-1 as an alternative target of Rad6-Rad18 ubiquitination. Indeed, both 9-1-1 and its clamp loader are required for Rad6-Rad18 mediated DNA damage induction.

### 3.3.2. Rad6-Rad18 and the Eukaryotic SOS Response

The roles of Rad6-Rad18 in gene regulation in response to DNA damage is reminiscent of the bacterial SOS response, in which RecA functions as a key regulator that is activated through binding to ssDNA and catalyzes the autocleavage of the repressor LexA, leading to the derepression of more than 30 SOS regulon genes (Friedberg et al., 2006). Interestingly, like RecA, the Rad6-Rad18 complex has also been reported to possess ssDNA binding and ATPase activities, as well as a ubiquitin conjugating activity (Bailly et al., 1997). Indeed, our microarray data indicate that *RAD6* and *RAD18* are coordinately required for the DNA damage induction of several hundred genes.

In a broad sense, *E. coli* RecA controls three important cellular responses to DNA damage, namely homologous recombination via RecBCD and RecFOR, TLS by working with PolIV and PolV, and the SOS response that coordinately provide a survival mechanism when cells encounter replication blocks (Figure 3-14). Our findings presented in this report, along with previous reports, argue that the Rad6-Rad18 complex assumes most if not all RecA functions to coordinate such broad cellular responses. Firstly, Rad6-Rad18 as an E2-E3 ubiquitination complex mono-ubiquitinates PCNA to promote Polζ and Polη mediated TLS (Hoege et al., 2002; Stelter and Ulrich, 2003). Secondly, this mono-Ub-PCNA is required for PCNA poly-ubiquitination via a Lys63 chain linkage for an error-free mode of DNA damage tolerance (Hoege et al., 2002)

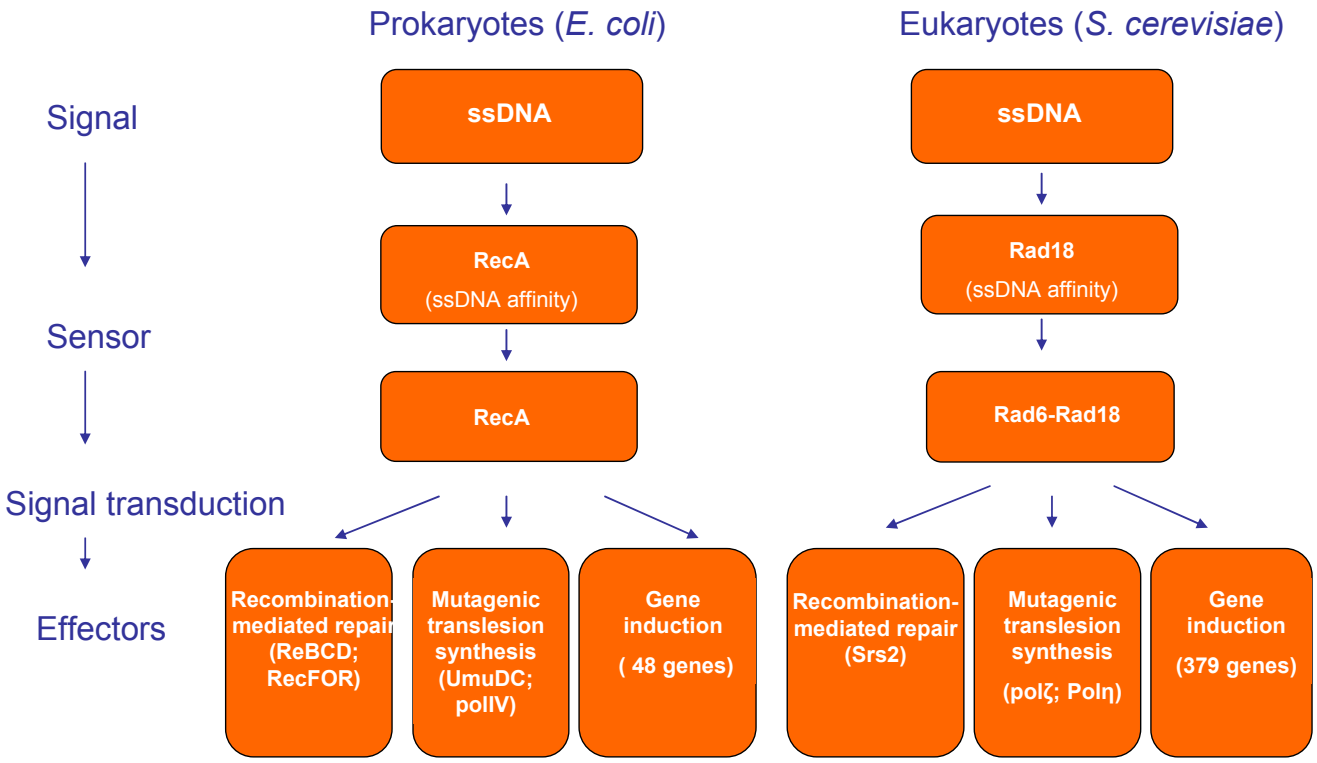



**Figure 3-14. The comparison between prokaryotic SOS response and putative SOS responses in eukaryote.**

reminiscent of the RecFOR activity in *E. coli* (Hanna et al., 2004). Thirdly, although Rad6-Rad18 does not have homologous recombinase activity like RecA, it may compete with the Ubc9-Siz1 complex that sumoylate PCNA at the same K164 residue; sumoylated PCNA recruits the DNA helicase Srs2 that inhibits the recombinase activity of the yeast RecA homolog Rad51 (Papouli et al., 2005; Pfander et al., 2005). Finally, data presented in this report demonstrate that Rad6-Rad18 also fulfills yet another core RecA activity in the SOS response via mono-ubiquitinating the Rad17 subunit of the 9-1-1 clamp (Figure 3-14).

There are several notable differences between bacterial and eukaryotic SOS responses. Firstly, unlike RecA, eukaryotic cells employ ubiquitination, a process not found in bacteria. Indeed, the ubiquitination activity of both Rad6 and Rad18 are absolutely required for all of the above functions including gene regulation. Secondly, while all SOS regulon genes share a common promoter element recognized by LexA, promoter analysis of genes induced by DNA damage and dependent on *RAD6-RAD18* did not reveal common elements (data not shown). As a matter of fact, among three sets of well-characterized yeast DNA damage inducible genes, their effectors and mechanisms of damage induction are completely different. The *RNR* genes share an X box sequence in their promoters that are bound by a Crt1 repressor and two co-repressors Tup1 and Ssn6; inactivation of any repressor results in complete derepression (Huang et al., 1998). *PHR1* is regulated by two repressors, Gis1 and Rph1; simultaneous inactivation of both repressors results in a synergistic *PHR1* depression (Jang et al., 1999). In contrast, *MAG1* and *DDI1* are divergently transcribed and share a promoter element recognized by an activator Pdr3 (Zhu and Xiao, 2004). Lastly, many DNA damage-inducible genes examined are variably regulated by cell cycle checkpoints (Aboussekhra et al., 1996;

Kiser and Weinert, 1996; Zhu and Xiao, 2001), which appear to converge at the point of Rad53 activation, whereas the downstream events are rather different. In the case of *RNR* genes, the Dun1 kinase activity is required for Crt1 phosphorylation and subsequent dissociation from *RNR* promoters (Huang et al., 1998). *MAG1* induction also requires *DUN1* (Zhu and Xiao, 2001), whereas *PHR1* induction is dependent on *RAD53* but does not require *DUN1* (Jang et al., 1999). Results presented in this report provide a mechanism in which the DNA damage signal recognized by the Rad6-Rad18 E2-E3 complex is relayed to the cascade of cell cycle checkpoints that have been previously regarded as a eukaryotic SOS response. Since all the genes discussed in this report are highly conserved in eukaryotes from budding yeast to humans, it is conceivable that the regulatory mechanism described in this report may apply to higher eukaryotes as well.

### 3.3.3. *RAD6-RAD18* and the Damage Checkpoint Pathway

The 9-1-1 clamp and the RFC-like clamp loader have been implicated as DNA damage sensors (Friedberg et al., 2006). Findings in this study that Rad6-Rad18 is required for Rad17 ubiquitination in a DNA damage-dependent manner place the Rad18 ssDNA binding protein as a strong candidate for a sensor in the damage checkpoint pathway. It is unclear whether the Rad24/9-1-1 and Rad6-Rad18 complexes independently recognize the damage site or one serves to recruit the other. Nevertheless, it is conceivable that once in proximity, Rad6-Rad18 will mono-ubiquitinate Rad17, which is an important signal for Rad53 phosphorylation and gene regulation.

Surprisingly, despite numerous efforts, *RAD6* and *RAD18* have not been implicated in the damage checkpoint pathway, which may be explained by several scenarios. Firstly, the 9-1-1 complex may be activated by alternative means, which may

undermine the contribution of Rad6-Rad18 in the damage checkpoint. Indeed, the Ddc1 subunit of 9-1-1 is phosphorylated in response to DNA damage (Longhese et al., 1997), which may be functionally redundant with Rad17 ubiquitination. Secondly, the Rad17 ubiquitination may be specifically responsible for gene regulation but not for cell cycle arrest, although it is unclear how this could be achieved. Thirdly, the checkpoint response of Rad6-Rad18 may be lesion-specific. Hence, the checkpoint defect of a *rad6* or *rad18* mutant may not be readily detected in experiments using UV and ionizing radiations as damage sources. Finally, the dual functions of Rad6-Rad18 in PRR and damage checkpoint may undermine detection of its checkpoint function, which is reminiscent of the response of the *rad9 rad52* double mutant to ionizing radiation (Weinert and Hartwell, 1988). In this case, lack of PRR activity may slow down S-phase progression in MMS-treated cells, which may undermine its damage checkpoint activity. With the above analysis in mind, we are confident that future experiments will be able to determine whether *RAD6* and *RAD18* are indeed involved in the damage checkpoint.

### 3.3.4. Coordination of DNA Damage Tolerance by Dual Ubiquitination of PCNA and 9-1-1

Perhaps the most striking finding in this report is the demonstration of 9-1-1 as a novel alternative ubiquitination target to PCNA. Both PCNA (Hoege et al., 2002) and 9-1-1 are mono-ubiquitinated in a *RAD6-RAD18* dependent fashion when cells are treated with MMS under similar experimental conditions. Hence, it is safe to conclude that the Rad6-Rad18 ubiquitination complex coordinates cellular tolerance to DNA damage via simultaneous ubiquitination of two DNA clamps (Figure 3-15). In this respect, it is of great interest to note that it was recently reported (Barbour and Xiao, 2003) that the

damage checkpoint pathway may function as a third branch within the PRR pathway with respect to tolerance of MMS-induced damage. In light of results presented in this report, it is now clear that the damage checkpoint pathway represented by *RAD9*, *RAD24* and 9-1-1 is not a branch of PRR but instead under the same umbrella of *RAD6-RAD18*. Hence, in the absence of PCNA ubiquitination and the presence of MMS-induced damage, the damage checkpoint via Rad17 ubiquitination provides a pivotal role in cell survival. It is also interesting to note a recent report (Sabbioneda et al., 2005) that the 9-1-1 checkpoint clamp physically interacts with Polζ and is involved in Polζ-mediated mutagenesis. Perhaps like its PCNA counterpart (Bienko et al., 2005; Kannouche et al., 2004), Rad17 mono-ubiquitination may enhance its affinity for TLS polymerases. Taken together, the Rad6/Rad18 complex is an excellent candidate for the central regulator that coordinates eukaryotic cellular response to DNA damage, including damage tolerance, damage checkpoint as well as an SOS-like transcriptional regulation.

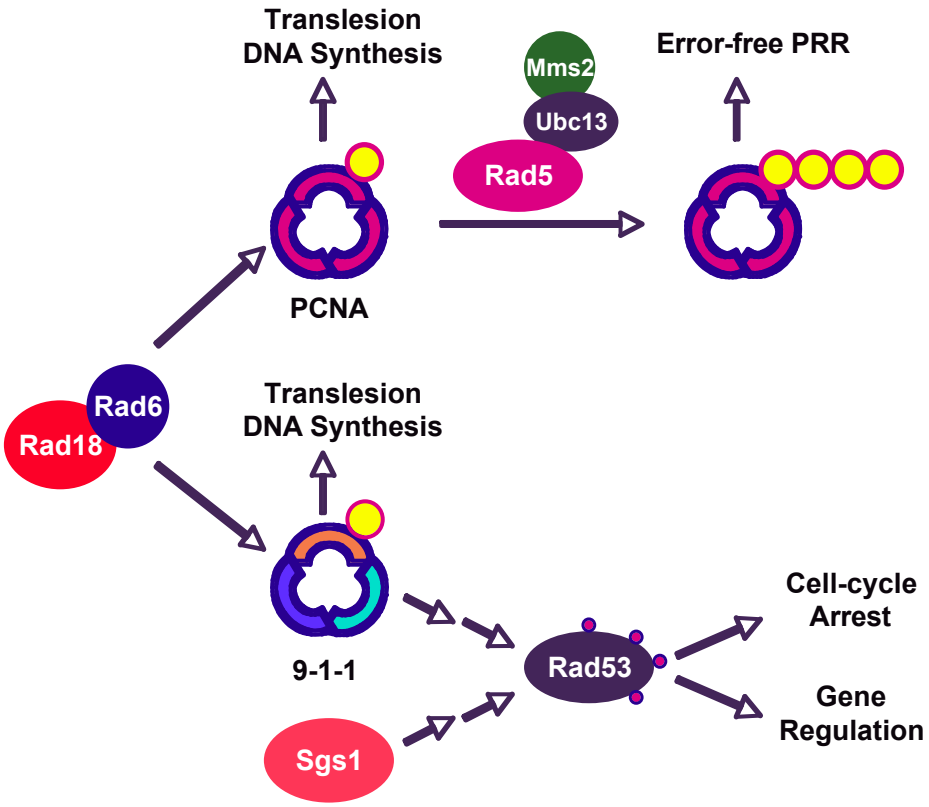



**Figure 3-15. A Model Depicting Coordinated Regulation of DNA Damage Tolerance through Rad6-Rad18 Mediated Mono-ubiquitination of Two DNA Clamps.** In response to DNA damage, the Rad6-Rad18 ubiquitination complex mono-ubiquitinates both Pol30 (PCNA) and the Rad17 subunit of 9-1-1. Ubiquitinated PCNA and possibly 9-1-1 recruit translesion polymerases to bypass the replication-blocking lesion. PCNA can be further poly-ubiquitinated by the Rad5-Ubc13-Mms2 complex, whereas mono-ubiquitinated 9-1-1 activates the damage checkpoint pathway leading to Rad53 phosphorylation, which results in transcriptional regulation and cell cycle arrest.

# CHAPTER FOUR

## TWO IDENTICAL MMS-INDUCIBLE GENES IN *SACCHAROMYCES CEREVISIAE*

### 4.1. Introduction

In Chapter Three of this thesis, the Rad6/Rad18 complex was introduced as a DNA damage sensor to mediate an eukaryotic SOS response. For the investigation of the molecular mechanisms of DNA damage induction in *S. cerevisiae*, the study on how ultimate effectors execute the regulation of transcription is also an important issue to understand this basic biological process. Therefore, considerable effort was also spent to determine the *cis*-acting elements and *trans*-acting factors involved in the DNA damage induction of some specific genes.

As brought forth in the introduction, most of the DNA repair genes are expressed at a low level under normal growth conditions and their expression is induced upon exposure to DNA damaging agents. For example, in *E. coli*, approximately 48 genes are co-ordinately induced after UV irradiation (Courcelle et al., 2001; Walker, 1985). In *S. cerevisiae*, at least 30 genes are known to be DNA damage inducible and their regulation has been well studied (Bachant and Elledge, 1998; Friedberg et al., 2006). The gene regulation and biochemical pathways involved in responding to DNA damage in mammalian cells share many features with those of *S. cerevisiae*, and many of DNA damage-inducible genes in mammalian cells have been linked to cancer (Criswell et al., 2003; Friedberg et al., 2006; Weinert, 1997).

During last decade, with the development of microarray technology, significant progress has been made towards the understanding of DNA damage induction. DNA microarray technology enables simultaneous examination of how the entire genome responds to DNA damaging agents. In a microarray analysis, 325 budding yeast genes were reported to increase more than 4-fold at the transcript level after exposing cells to the alkylating agent MMS (Jelinsky and Samson, 1999). The protein products of these 325 genes are involved in different biological functions, such as stress response, detoxification, DNA repair, DNA replication, cell cycle, signal transduction, cell wall biogenesis, protein degradation and membrane transport (Jelinsky and Samson, 1999). These genes are likely to protect yeast cells from the damage caused by MMS treatment, although the reasons why some genes are induced are difficult to rationalize.

To date, with the exception of a few well documented DNA-damaged inducible genes (e.g. *RNR2*, *RNR3*, *MAG1* and *PHR1*), it still remains unclear how these genes are regulated at the transcriptional level in response to MMS treatment. Results from previous studies suggest that upstream *cis*-acting regulatory elements and corresponding *trans*-acting factors may control MMS-induced transcription. For instance, *RNR3* is one of the genes most highly induced by MMS in *S. cerevisiae*. The induction of *RNR3* after MMS treatment is due to the removal of a transcriptional repressor protein known as Crt1 from the upstream *cis*-acting element X-box in the *RNR3* promoter (Huang et al., 1998). After MMS treatment, a checkpoint kinase cascade including Mec1, Rad53, and Dun1 protein kinases is activated and phosphorylates Crt1, resulting in the derepression of Crt1 target genes (Huang et al., 1998). Another MMS-inducible gene *MAG1* relies heavily on several *cis*-acting regulatory elements, upstream acting sites (UAS) and upstream repressing sites (URS), and the *trans*-acting factor Pdr3 to regulate its expression level

(Liu and Xiao, 1997; Xiao et al., 1993; Zhu and Xiao, 2004). Furthermore, the DNA damage induction of *MAG1* is also affected by checkpoint mutants such as *mec1*Δ, *rad53*Δ and *dun1*Δ (Zhu and Xiao, 1998; Zhu and Xiao, 2001). However, the promoters of these MMS-inducible genes do not appear to share common *cis*-elements and no consensus *trans*-acting factors that act on all these genes have been identified.

Here, we described two identical genes *DDI2* and *DDI3*, which are highly induced by MMS treatment. In the present study, we analyzed their promoter region, and identified several *cis*-acting regulatory regions that regulate the transcription in response to MMS treatment.

## 4.2. Results

### 4.2.1. Identification of *DDI2* and *DDI3*

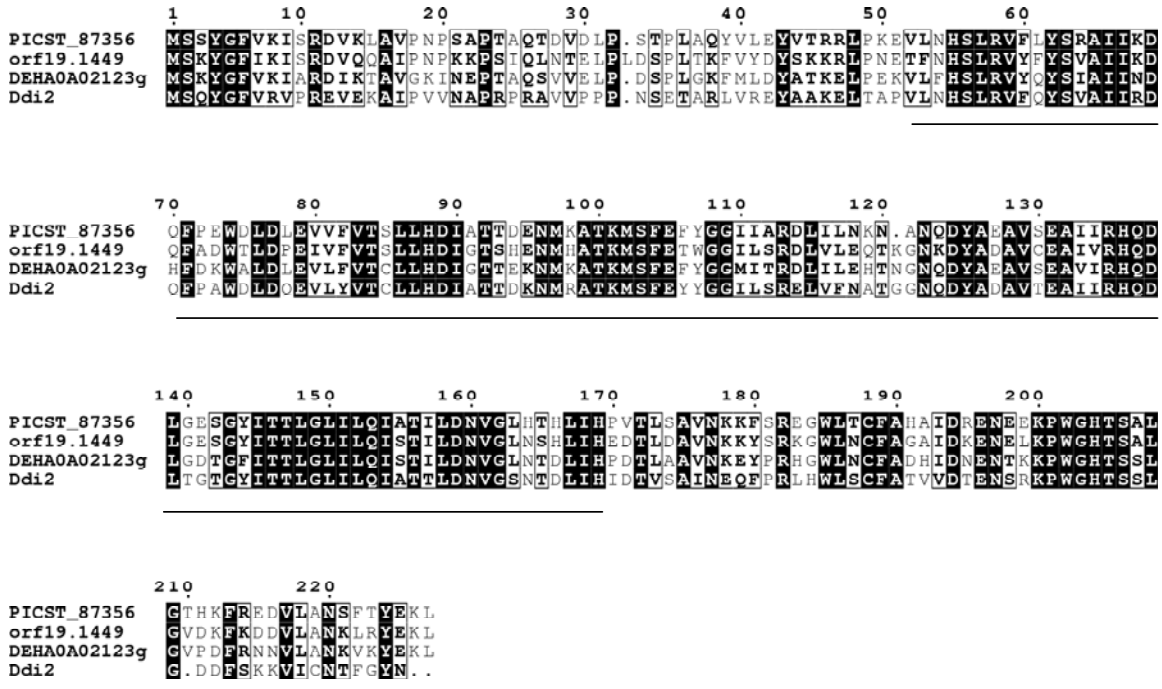
MMS is a DNA alkylating agent known to react with DNA at N and O atoms (Beranek, 1990; Friedberg et al., 2006). The O-alkylated bases (for example, $O^6$-methylguanine) are highly mutagenic (Beranek, 1990), while the N-alkylated purines (for example, $N^3$-methyladenine) inhibit DNA synthesis and require repair for proper replication (Chang et al., 2002). We previously performed a study where *S. cerevisiae* cells were treated with 0.1% MMS for 48 minutes and the global transcriptional response was assessed by microarray analysis. During this microarray analysis, two genes, *YFL061w* and *YNL335w*, showed the highest induction. After MMS treatment, the transcription of these two genes increased by 108-fold and 138-fold, respectively. Thus, we designated *YFL061w* as *DDI2* and *YNL335w* as *DDI3* for DNA damage inducible gene. Interestingly, *DDI2* and *DDI3* are two identical genes with exactly the same

promoter regions, but they are located on different chromosomes (SGD, www.yeastgenome.org, Stanford University).

*DDI2*/*DDI3* encodes a putative 225 amino acid protein, and the predicted Ddi2/Ddi3 contains an HD domain [named HD due to the conserved doublet of predicted catalytic residues (Histidine-Aspartic acid) in the predicted phosphohydrolases] (Figure 4-1A). HD domains are found in a superfamily of enzymes with metal-dependent phosphohydrolase activity (Aravind and Koonin, 1998). These enzymes appear to be involved in nucleic acid metabolism, signal transduction and possibly other functions in bacteria, archaea and eukaryotes. No polypeptide sequence in the worm, mouse or human genome database shows homology to Ddi2/Ddi3. However, close homologs of Ddi2 are identified in *Debaryomyces hansenii* (DEHA0A02123g), *Pichia stipitis* (PICST_87356) and *Candida albicans* (orf19.1449) (Figure 4-1 A). Interestingly, some predicted or known urea hydrolyase (cyanamide hydratase) in fungi show significant similarity to Ddi2 (Figure 4-1B).

In order to validate the microarray result, we performed Northern blot analysis to determine the transcriptional level of *DDI2*/*DDI3* after MMS treatment. The Northern blot result (Figure 4-2A) demonstrated that the transcription of *DDI2/DDI3* increased by 110-fold after MMS treatment. Furthermore, we isolated *DDI2* and its promoter (-709 to +1), and made a *lacZ* fusion construct. As expected, the β-gal assay also showed more than 100-fold induction of *DDI2-lacZ* by MMS treatment (Figure 4-2B). Taken together, *DDI2* and *DDI3* are two identical genes that can be highly induced by MMS.

A


PICST_87356
orf19.1449
DEHA0A02123g
Ddi2

B

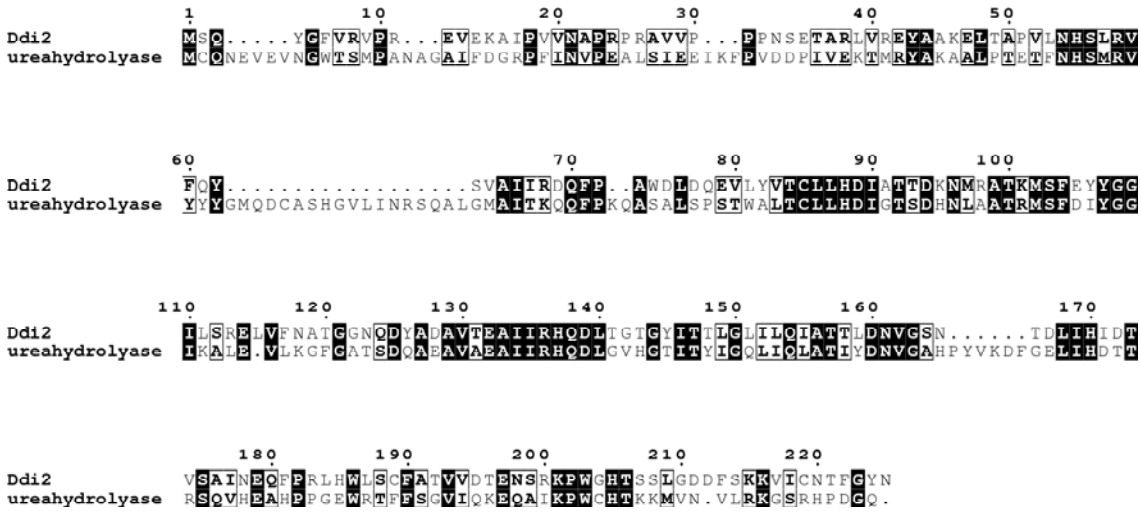

Ddi2
ureahydrolyase

**Figure 4-1. Protein sequence alignment of *DDI2*/*DDI3* and their homologs. (A)** Amino acid sequence comparison of *S. cerevisiae* Ddi2/Ddi3 and its putative homologs in *Debaryomyces hansenii* (DEHA0A02123g), *Pichia stipitis* (PICST_87356) and *Candida albicans* (orf19.1449). (**B)** Sequence comparison of Ddi2 and urea hydrolyase from *Aspergillus fumigatus*. All alignments were performed using the CLUSTALW program in EBI with additional manual modifications with ESPript software. The HD domain is underlined.

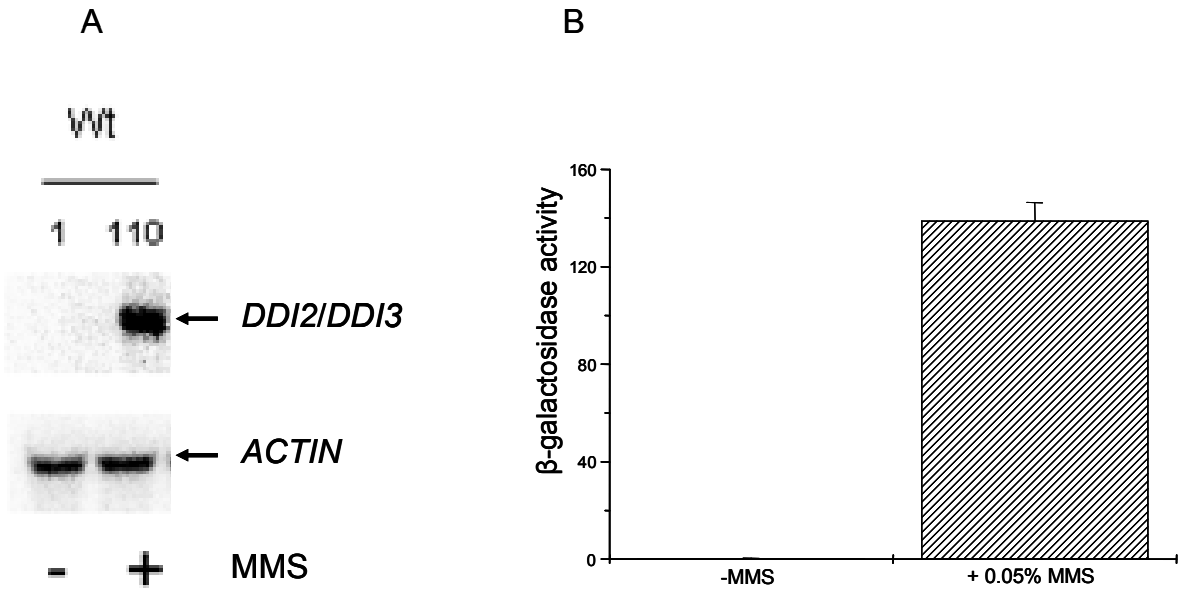


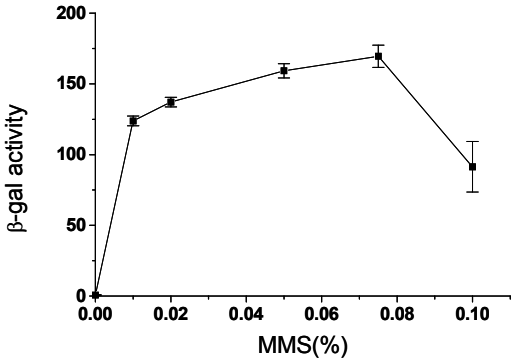

**Figure 4-2. The transcription of *DDI2/DDI3* is highly induced by MMS treatment.** (**A**) Northern blot analysis of *DDI2/DDI3*. Yeast cells DBY747 were treated with 0.1% MMS for 48 minutes prior to RNA isolation and Northern hybridization. Each lane contains 15 μg of total RNA. The blot was stripped and hybridized with an *ACT1* probe. The *DDI2* transcript level in each sample was normalized with reference to that of *ACT1*, and expressed as value relative to an untreated wild-type sample in the same blot. (**B**) The expression of *DDI2-lacZ* with or without MMS treatment. Log-phase cells were either treated with 0.05% MMS for 4 hours or without treatment prior to β-gal assays. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

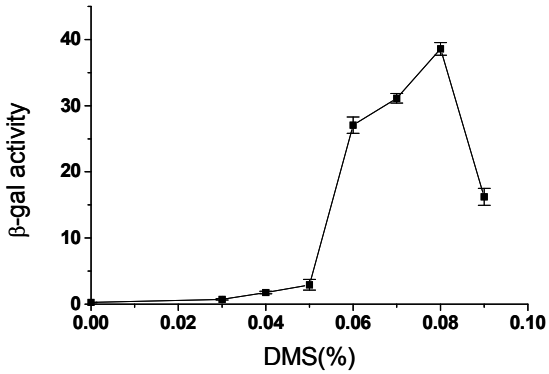
### 4.2.2. *DDI2* is only highly induced by $SN_2$ alkylating agents

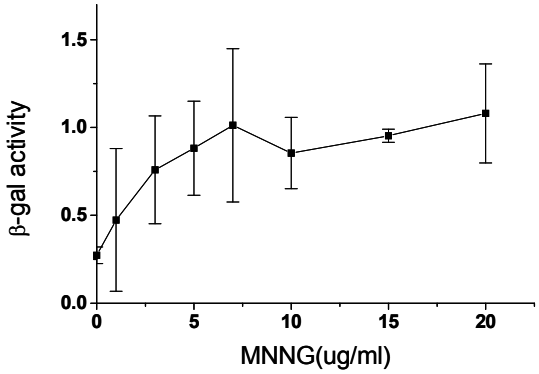
Since all of the well-documented DNA damage-inducible genes in budding yeast seem to respond to a wide spectrum of DNA damaging agents, we examined the inductionof *DDI2-lacZ* after treating yeast cells with different DNA damaging agents. To our surprise, the expression of *DDI2-lacZ* was only highly induced by $SN_2$ alkylating agents MMS and dimethyl sulfate (DMS) up to 322-fold and 150-fold, respectively. These compounds alkylate predominantly at nitrogens rather than the oxygens in DNA bases (Figure 4-3). The $SN_1$ alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which efficiently alkylates both nitrogens and oxygens, did not induce the expression of *DDI2-lacZ* (Figure 4-3). Other DNA damaging agents, such as ethyl methanesulfonate (EMS), γ-ray and hydroxyurea (HU), only mildly induced the expression of *DDI2-lacZ* by approximately 8-fold (Figure 4-3).

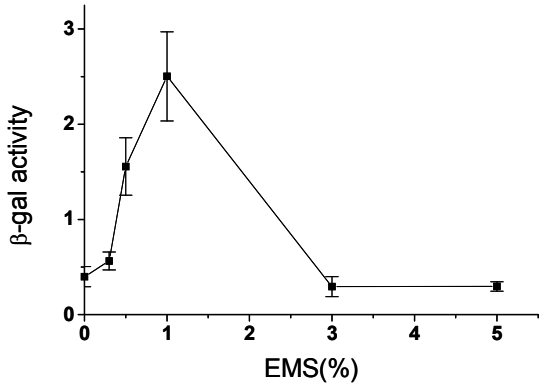
### 4.2.3. *DDI2-lacZ* upstream deletions identify UAS and URS

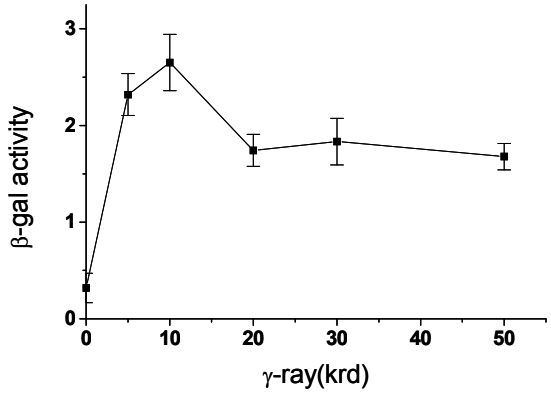
In order to identify UAS and URS elements in the *DDI2* promoter region, various deletions were introduced into the plasmid YEpDDI2-lacZ. Beta-gal activities of yeast transformants harbouring these plasmids were determined with or without MMS treatment. Therefore, any changes in the level of β-gal activity probably reflected the functions of the deleted sequences. As shown in Figure 4-4, deletion of the -709 to -358 regions increased the basal level of expression by 3-fold and MMS-induced expression by 1.7-fold compared to the intact promoter. This indicated the presence of upstream repressing sites in this region. Deletion of the region extending from -358 to -229 reduced


β-gal activity
MMS(%)
0
50
100
150
200
0.00
0.02
0.04
0.06
0.08
0.10


β-gal activity
DMS(%)
0
10
20
30
40
0.00
0.02
0.04
0.06
0.08
0.10


β-gal activity
MNNG(ug/ml)
0.0
0.5
1.0
1.5
0
5
10
15
20


β-gal activity
EMS(%)
0
1
2
3
0
1
2
3
4
5


β-gal activity
γ-ray(krd)
0
1
2
3
0
10
20
30
40
50

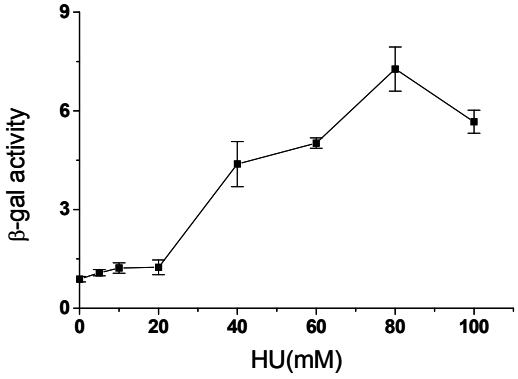

β-gal activity
HU(mM)
0
3
6
9
0
20
40
60
80
100

**Figure 4-3. The induction of *DDI2-lacZ* by different DNA damaging agents.** Log-phase cells were either treated by different chemicals with various concentrations for 4 hours or without treatment prior to β-gal assays. All the results are in Miller units and represent the average of at least three experiments with standard deviations.

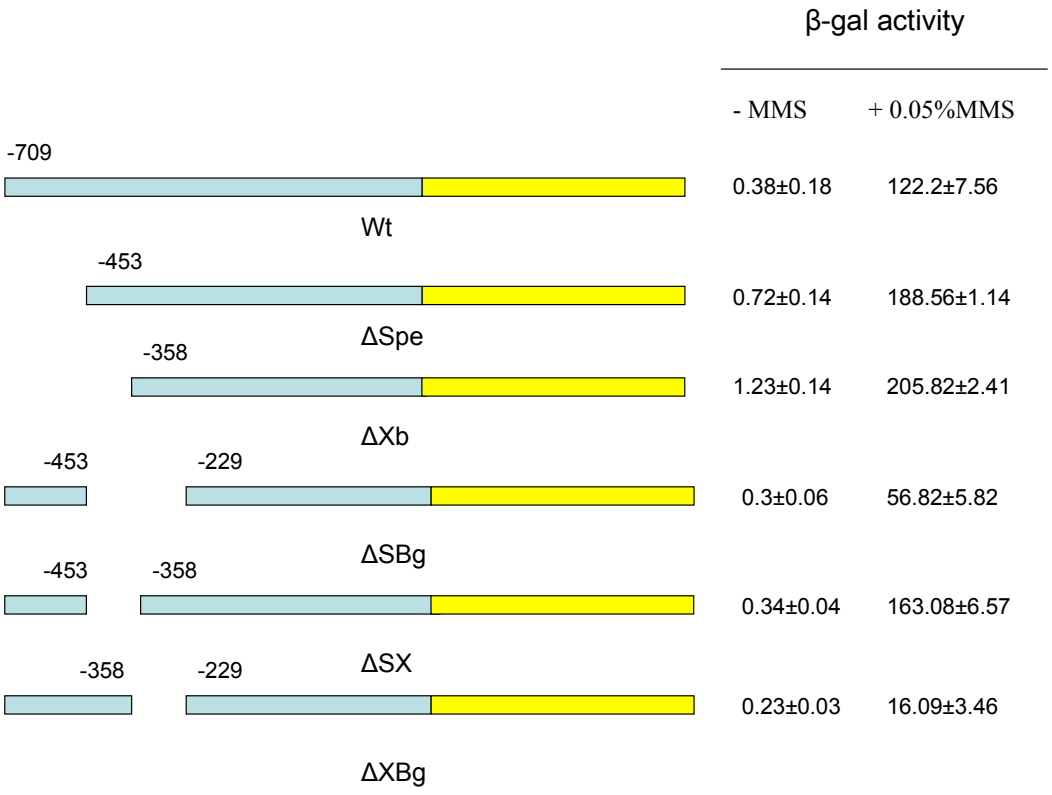



**Figure 4-4. The effects of deletions in *DDI2* promoter region on *DDI2* expression as monitored by β-galactosidase activity.** For each plasmid construct, the blue solid bar indicates the promoter region, while deletions are indicated by interruptions in the blue solid bar. The number indicates the distance to the start codon (ATG) of *DDI2*. β-gal activities are expressed in Miller units and represent the average of at least three experiments with standard deviations.

basal-level *DDI2-lacZ* expression by 1.7-fold and MMS-induced expression by 7.6-fold (Figure 4-4). Furthermore, when this region was kept intact (deletion of the -453 to -358 region), both the basal level and inducible expression of *DDI2-lacZ* were restored to wild type levels (Figure 4-4). These observations suggest that the region extending from -358 to -229 functions as a UAS in the *DDI2* promoter. We designated this region as $UAS_{DDI2}$.

### 4.2.4. The $UAS_{DDI2}$ confers activation and MMS inducibility to a heterologous promoter

To determine if the $UAS_{DDI2}$ is able to function in the context of a heterologous promoter, the $UAS_{DDI2}$ sequence was inserted into the promoter region of the *CYC1-lacZ* reporter gene in pLG669Sm (Guarente and Hoar, 1984). The *CYC1* promoter alone exhibited no MMS inducibility, and the β-galactosidase activity of *CYC1-lacZ* was reduced by 1.55 fold following MMS treatment (Figure 4-5). Insertion of the $UAS_{DDI2}$ into the *CYC1* promoter increased β-galactosidase activity by 1.77-fold in the absence of MMS induction. Furthermore, in contrast to *CYC1-lacZ*, which showed reduced β-galactosidase activity following MMS treatment, the insertion of $UAS_{DDI2}$ conferred a 2.77-fold MMS inducibility (Figure 4-5).

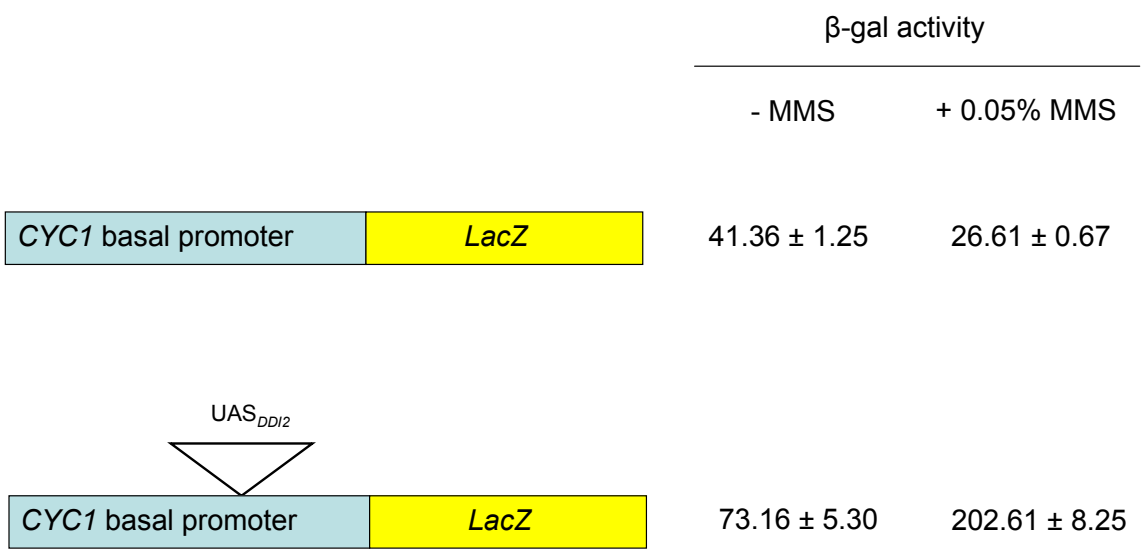



**Figure 4-5. Effects of the UAS$_{DDI2}$ on the heterologous *CYC1* promoter.** The left panel shows promoter the constructs used in this work. The right panel shows β-galactosidase activity of corresponding DBY747 transformants with or without MMS treatment. β-gal activities are expressed in Miller units and represent the average of at least three experiments with standard deviations.

## 4.3. Discussion

In the present work, we report the identification of two identical genes, *DDI2* and *DDI3*, which can be highly induced by MMS. The induction ratio reaches up to 322-fold in the β-gal assay, and it is by far the highest DNA damage induction ratio known in budding yeast.

In budding yeast, a feature of the DNA damage responses is that most DNA damage-inducible genes analyzed to date can be induced by a variety of DNA damaging agents, (Liu and Xiao, 1997). For instance, DNA repair genes *PHR1*, *MAG1*, *RAD2* are induced by MMS, MNNG, UV irradiation, 4NQO or γ-rays (Chen and Samson, 1991; Robinson et al., 1986; Sebastian et al., 1990), irrespective of whether the gene product is involved in the repair of the specific damage. *RNR* genes, a protein-degradation related gene *UBI4*, and the putative S-phase checkpoint gene *DDI1* can also be induced by different DNA damaging agents, such as UV irradiation, 4NQO, HU, MNNG, and MMS (Elledge and Davis, 1990; Liu and Xiao, 1997; Treger et al., 1988). To our surprise, *DDI2*/*DDI3* can only be highly induced by the $SN_2$ alkylating agents MMS and DMS, slightly induced by EMS, HU and γ-rays, and not induced at all by the $SN_1$ alkylating agents MNNG. Therefore, it implies that the signal for high level induction of *DDI2/DDI3* by MMS could be generated by specific substrates formed by MMS treatment. It is known that MMS and DMS induce a much higher amount of 1-methyladenine in DNA than MNNG and MNU (Singer and Grunberger, 1983). Meanwhile, the enhanced transcriptional level of *DDI2/DDI3* after MMS treatment is postulated to protect yeast cells against the damage caused by MMS. Thus, the protein product of *DDI2/DDI3* might be involved in the repair of 1-methyladenine directly or indirectly.

Analysis of the *cis*-acting regulatory elements in the *DDI2* promoter region allows us to obtain some clues as to how this gene is regulated. Since deleting the region between -358 and -229 ($UAS_{DDI2}$) decreases both basal-level expression and the induction by MMS, our data suggest that this region is required for transcriptional activation. Given that pLG669Sm does not contain native $UAS_{CYC1}$ (Guarente and Hoar, 1984; Guarente et al., 1984), the observation that $UAS_{DDI2}$ alone conferred a 1.77-fold increase in β-gal activity in a heterologous *CYC1-lacZ* promoter without treatment further confirms that it is a UAS and directly influences basal transcription. Moreover, the insertion of $UAS_{DDI2}$ caused a 2.77-fold induction of *CYC1-lacZ* following MMS treatment, indicating that $UAS_{DDI2}$ is required for MMS inducibility.

A comparison of sequences within the promoter regions of some damage-inducible genes, such as *RAD2*, *RAD51*, *MAG1*, *RNR2* and *RNR3*, have identified the presence of a consensus 8-bp sequence [5'-GG(T/A)GGCGA-3'] (Elledge and Davis, 1989; Liu and Xiao, 1997; Siede et al., 1989; Xiao et al., 1993). This consensus sequence could be responsive to DNA-damage induction (Liu and Xiao, 1997). However, no such consensus sequence was found in the $UAS_{DDI2}$ region. Instead, we noticed that a 6 bp sequence "AAAAGA" is repeated four times in this 129 bp region. Three of them are in one direction, and one is in the opposite direction. Although this 6-bp sequence shares no significant sequence homology with any reported upstream activating sequences in DNA damage inducible genes, it is unlikely that these repeats appear by coincidence.

Compared with the intact promoter, deletion of the region between -709 and -358 increased both basal level and induced expression. This observation indicates the presence of URS in this region. Moreover, deletion of the region between -453 and -229 resulted in higher induced expression than the deletion of $UAS_{DDI2}$. This suggests that

there may be a URS in -453 to -358 region. Meanwhile, it suggests that the repression function of URS in -453 to -358 region is independent of the $UAS_{DDI2}$. Since extended deletion in the -453 to -709 region resulted in increased MMS-induced expression, it is likely that there is more than one URS in the region between -709 and -358.

According to these results, we propose a model explaining the control of *DDI2* expression. The expression of *DDI2* is positively regulated by the $UAS_{DDI2}$. It is possible that the 6-bp repeats are recognized by *trans*-acting factors, and the binding of the factors to this region can significantly activate transcription. The MMS treatment might enhance the binding of these factors so as to obtain high level induction. The region between -709 and -358 is involved in a negative regulation. However, the transcription repression carried out by the URS is not as strong as the transcription activation by the UAS. Consequently, it is unlikely that the mechanism of high level induction is mainly due to the competition of transcription factors for the URS and the UAS, even though the UAS and URS regions are close to each other in the putative promoter region.

# CHARTER FIVE

# CRT10 IS A NOVEL REGULATOR OF *SACCHAROMYCES CEREVISIAE* RIBONUCLEOTIDE REDUCTASE GENES

## 5.1. Introduction

In addition to the general DNA damage sensor and the *cis*-acting elements in the promoter regions introduced in the previous chapters, the *trans*-acting factors are also important for the study of DNA damage induction. In this chapter, we will introduce a novel *trans*-acting factor that regulates the expression of yeast ribonucleotide reductase genes, which are known to be induced by DNA damaging agents.

Ribonucleotide reductase (Rnr) catalyzes the rate-limiting steps in dNTP synthesis. Three classes of Rnr have been identified (Jordan and Reichard, 1998). Class I enzymes, which are found in all eukaryotes and some prokaryotes, consist of an $\alpha_2\beta_2$ tetramer made up of two large ($\alpha$) and two small ($\beta$) subunits. The $\alpha$ subunit possesses binding sites for substrate and allosteric effectors, and the $\beta$ subunit contains a binuclear iron complex that interacts with a specific tyrosine residue to form a tyrosyl free radical and is essential for Rnr activity (Eklund et al., 2001; Fontecave et al., 1992). In the budding yeast *Saccharomyces cerevisiae,* the large Rnr subunit is encoded by two highly homologous genes, *RNR1* and *RNR3* (Elledge and Davis, 1990). *RNR1* is an essential gene, whereas *RNR3* is nonessential. *RNR1* transcription is tightly regulated during the cell cycle and moderately induced by DNA damage, whereas *RNR3* is barely transcribed under normal conditions but is highly inducible by DNA damage, increasing up to 100-fold (Elledge and Davis, 1990). The small Rnr subunit is encoded by *RNR2* and *RNR4*, both of which

are essential and DNA damage inducible (Elledge and Davis, 1987; Huang and Elledge, 1997; Hurd et al., 1987), although *RNR4* null mutants in some yeast strains appear to be viable (Baskaran et al., 1997).

The tight regulation of Rnr during the cell cycle and by DNA damage is thought to be crucial for the maintenance of balanced dNTP pools for high-fidelity DNA replication and repair (Elledge et al., 1993; Zhou and Elledge, 1992). Failure to provide a sufficient and balanced dNTP pool may cause misincorporation of dNTPs into DNA, which in turn results in genetic abnormalities and cell death (Chabes et al., 2003). The regulation of Rnr involves multiple mechanisms in budding yeasts, including transcriptional regulation (Longhese et al., 2003), protein (Zhao et al., 2001) and allosteric (Chabes et al., 2003; Reichard et al., 2000) inhibition and subcellular localization (Yao et al., 2003). The DNA damage-induced transcriptional activation is mediated by the cell cycle checkpoint genes. The stalling of the replication fork or DNA damage triggers a DNA damage checkpoint pathway composed of the protein kinase cascade Mec1, Rad53 and Dun1 (Zhou and Elledge, 1993). Activated Dun1 phosphorylates a Crt1 repressor, and hyper-phosphorylated Crt1 no longer binds the X-box sequence found in the promoters of *RNR* genes, resulting in transcriptional derepression (Huang et al., 1998).

A second mechanism is Sml1-dependent. Sml1 inhibits the yeast Rnr activity by binding its larger subunit (Chabes et al., 1999; Zhao et al., 2000; Zhao et al., 1998). Activated Sml1 levels decrease at S phase and after DNA damage, resulting in derepression of Rnr activity (Zhao et al., 2001). The inactivation of Sml1 is caused by post-transcriptional regulation and also requires Mec1-Rad53-Dun1-dependent phosphorylation (Zhao et al., 2001; Zhao and Rothstein, 2002), which again testifies to

the need for tight Rnr regulation. The tight regulation of Rnr activity appears to be true for other organisms, such as fission yeast (Mirza et al., 2003), indicating that such regulation is evolutionarily conserved.

It is anticipated that additional genes and/or mechanisms may be involved in the regulation of Rnr activities. To investigate this possibility, we utilized the powerful budding yeast genetic system to identify such genes, and report here the identification of a novel gene, *CRT10*, whose mutation enhances hydroxyurea (HU) resistance. Genetic characterization indicates that *CRT10* is involved in the transcriptional regulation of *RNR* genes.

## 5.2. Results

### 5.2.1. Identification of *CRT10*

HU is a potent inhibitor of Rnr. Inhibition of Rnr leads to depleted dNTP pools, the subsequent stalling of the replication forks and S phase cell-cycle arrest (Eklund et al., 2001; Slater, 1973). In order to identify *S. cerevisiae* genes whose mutations alter cellular sensitivity to HU, we performed an HU resistance screen with a haploid yeast mutant library consisting of 4850 individual gene deletion strains. Among HU-resistant mutants, the *YOL063c* deletion mutant displayed significant resistance to HU and this gene has not been previously characterized. *YOL063c* encodes a putative 957-amino acid, 109 kDa protein and was designated as *CRT10*, after the nine previously described putative *CRT* (*c*onstitutive *R*NR *t*ranscription) regulator genes (Zhou and Elledge, 1992).

The predicted Crt10 contains leucine repeats at residues 105-145 with a sequence L-$X_9$-L-$X_8$-L-$X_6$-L-$X_6$-L-$X_6$-L, a putative transmembrane domain at residues 191-206, and one copy of the Trp-Asp (WD) repeat motif at residues 253-267 (van der Voorn and

Ploegh, 1992) (Figure 5-1). WD-repeat proteins are found in all eukaryotes and implicated in a wide range of crucial functions. These proteins typically contain 4-16 copies of the WD motif (Smith et al., 1999); however, only one WD repeat motif was found in Crt10.

A database search with the Crt10 protein sequence revealed several homologous sequences in other organisms. The closest homologs are found in members within the *Saccharomyces* family**.** In addition, a putative protein (ADR329Wp) in *Eremothecium gossypii* and *Candida albicans* hypothetical protein (CAG58307.1) show significant homology to Crt10; a hypothetic protein (SPBC27B12.05) from *Schizosaccharomyces pombe* also shows limited homology to Crt10 (data not shown). No polypeptide sequence in the worm, mouse or human genome database has significant similarity to Crt10, suggesting that Crt10 may be unique to lower eukaryotes, possibly within unicellular eukaryotic microorganisms.

### 5.2.2. Deletion of *CRT10* enhances survival of the *mec1*Δ mutant

The *crt10* mutant was originally isolated for its enhanced resistance to HU killing in a library screen. We compared the *crt10*Δ mutant to its isogenic wild-type strain BY4741 and found that it indeed displayed an enhanced resistance to HU (Figure 5-2A).

In order to rule out the possibility that this *crt10* strain contains additional unknown mutation(s), we made a *crt10*Δ*::LEU2* deletion cassette and created a *crt10* null mutant in a different strain background. As shown in Figure 5-2B, targeted deletion of *CRT10* also resulted in a similar HU-resistant phenotype. Deletion of *CRT10* led to slightly enhanced resistance to MMS, but not to UV (data not shown), suggesting that Crt10 probably functions specifically in a pathway in response to either DNA replication

MPPQIPNENDDLFTRWLKSRAIIQRAVSTRECFDSEVFLASGGWNITNEIITLKKYYQLK 60
WPNSSCNSFHPKTVEFIKERLHNLEEHDSSWKIPNPAYSFKKAFLEDTKSAFSNLEPVWG 120
PSRLLNPAELLLPQDEKLLVQEIPLEFAPFQYTNRFAYGGLQFKNNLFVTYGSYSFLAAG 180
QCVEVHNFDI**LLNVSSLEICHALLPV**IIPDDGDVRNFRNSSYVKFKDTQFNSIPELCSIN 240
FMKICNFMHQDF***LLACGDNGIVYIWEI***NKVIKIFNKFTSDILGGKDNSRERYINVDPYMV 300
LRVEESCWSVDVIDINGIIYIAVGHNKPGVTVFAFDKDVKKERRYIRPLDLPSSHNVPCV 360
NFVPNSKDSVGYITLSYCSIFGNVVTVKLKEHDCTILTSFLDTQFFGDDLWTITPLTKKD 420
FAKVDNFELLNLNYQDGFKESMLYSICRDDFLLGYYCDNAYLSGNFGIGTLLNQFQVPVT 480
DLRLTSSAGIPDEVIPLRFTSFDRNYTTTGSIKYEYSREDFALILHAGDLDDMNDAVTKN 540
TSCEQHLHQWTFWEDSGYKHYRATERGFSKYKDIINTFPQLITPSGRNKTSQYQNTSGRK 600
ICEPSTYKLTDLENDIEDISREFNRSIRNLKMDKQRQLRTSKEFKSLSSVNHIPNIESGN 660
FLWYNTDAAADWRTLFGKDLNTVLKDPEICSLQLNSTEEDDVNSDPENEESGSSLTSFQR 720
RYRDTEQRAHLKSESQKSWGFHNYVRNVKRLLESAVPGSEDSPLGYQLSEMHDEFFFLTT 780
AHRLVLMKANPLIIISATHHEIFPLDGVVTCASKSLLQALNRINFVCHIKELNCIAVASQ 840
LGLISLLRLTEYRGIYSFRQEYILGWEVQDPVNPSPECRCNRNLFDAPMYGADGESSDTY 900
CGVCDVYFPMGDICGLDYTYASDSEELKRKGYATLYVASRGSLRAFKITTEHGTTQQ 957

**Figure 5-1. The deduced *S. cerevisiae* Crt10/YOL063c amino acid sequence.** The putative leucine repeat (underlined), transmembrane domain (bold) and WD repeat (bold and italicized) are indicated.

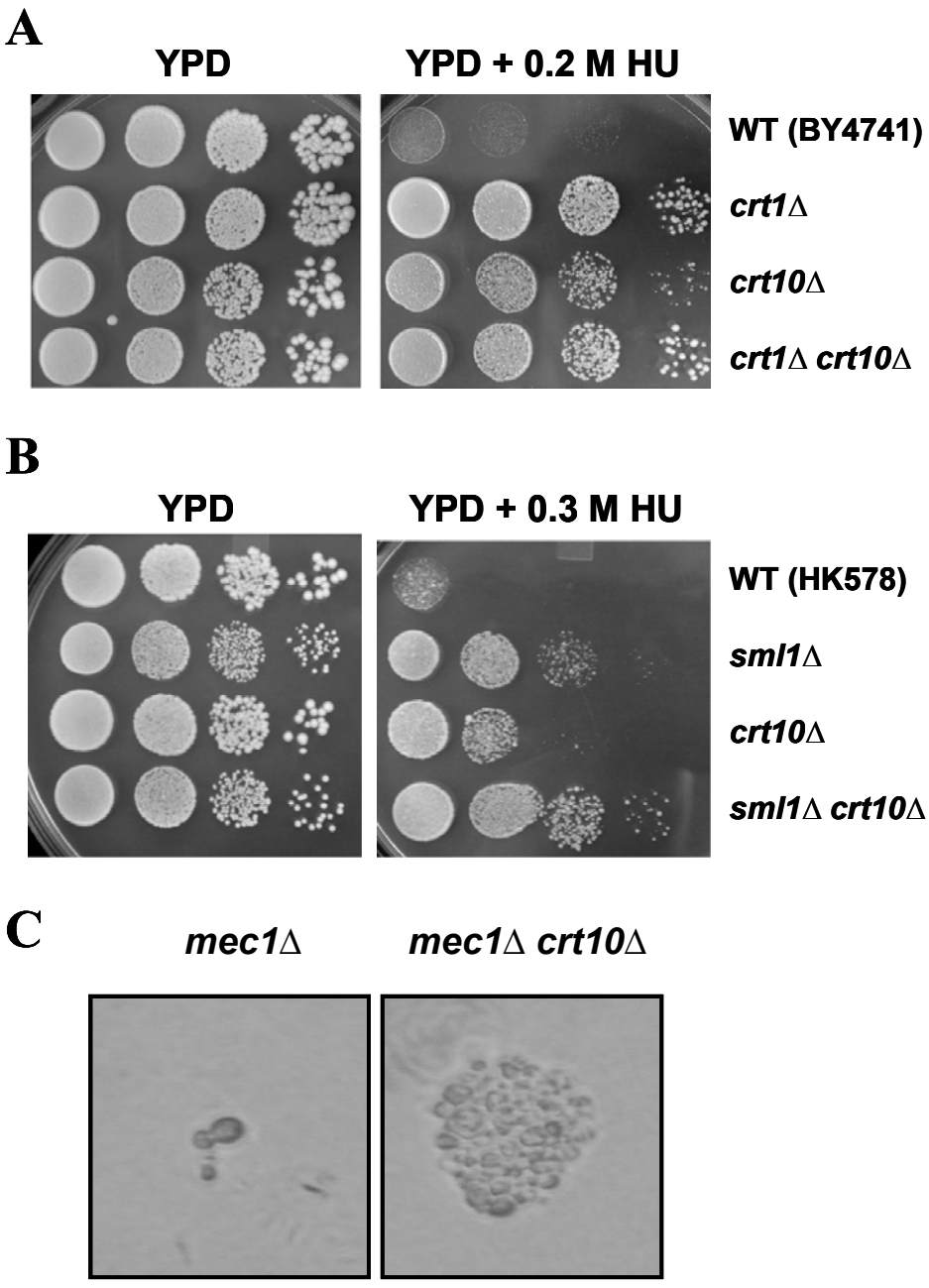

A
YPD
YPD + 0.2 M HU
WT (BY4741)
crt1Δ
crt10Δ
crt1Δ crt10Δ
B
YPD
YPD + 0.3 M HU
WT (HK578)
sml1Δ
crt10Δ
sml1Δ crt10Δ
C
mec1Δ
mec1Δ crt10Δ

**Figure 5-2. Genetic interaction of *CRT10* with *CRT1* (A), *SML1* (B) and *MEC1* (C).** **(A,B)** Epistasis analysis. Tenfold serial dilutions of log-phase cultures were spotted on YPD plates and on YPD plates containing HU as described. Plates were incubated for 3 days at 30$^{o}$C before photographing. Strains used: (A) BY4741 (WT), WX1152 (*crt10*Δ), WX1153 (*crt1*Δ), and WXY1154 (*crt1*Δ *crt10*Δ). (B) HK578-10A (WT), U952-3B (*sml1*Δ), WXY1157 (*crt10*Δ), and WXY1159 (*sml1*Δ *crt10*Δ). (C) The nonviability of *mec1*Δ is partially rescued by deletion of *CRT10.* Tetrads resulting from a cross of WXY1158 (*MEC1 SML1 crt10*Δ) and U953-61A (*mec1*Δ *sml1*Δ *CRT10*) were dissected and the growth of each spore was followed by microscopic analysis. The representative picture was taken after a 4-day incubation at 30$^{o}$C.

arrest or the endogenous nucleotide pool balance.

To distinguish the above two possibilities, we took advantage of a cell cycle checkpoint mutant, *mec1*. It is known that the *mec1* null mutant is nonviable; however, its nonviability is due to the decreased expression of *RNR* genes rather than the loss of checkpoint functions. Hence, its viability can be rescued by deletion of either the Rnr inhibitor gene *SML1* (Zhao et al., 1998) or the *RNR* repressor *CRT1* (Huang et al., 1998), or by overexpression of *RNR1* (Desany et al., 1998). We reasoned that if Crt10 acts upon Rnr expression/activity, deletion of *CRT10* may be able to rescue the *mec1*Δ nonviability, whereas if it acts upon a stalled replication fork, the *CRT10* deletion should not be able to rescue *mec1*Δ. The *mec1*Δ *sml1*Δ double mutant was crossed to *crt10*Δ in an isogenic background. Haploid spores recovered from 40 tetrads were genotyped by replica plating on appropriate media. No viable *crt10 mec1* double mutant colonies were obtained. However, under the microscope, it was found that the *mec1*Δ mutant cells did not extend beyond two cell divisions, whereas the *crt10*Δ *mec1*Δ double mutant cells formed microcolonies containing up to several hundred cells (Figure 5-2C). This is in contrast to the *sml1 mec1* double mutant from the same experiment, which formed visible colonies (data not shown). Hence, deletion of *CRT10* appears to rescue *mec1*Δ cells from immediate death.

### 5.3.3. *CRT10* belongs to the *CRT1* regulatory pathway

Since deletion of *CRT10* results in HU resistance and partially rescues the *mec1*Δ mutant, it is most likely involved in the regulation of Rnr activity. *CRT1* and *SML1* are two genes regulating Rnr by different mechanisms, the former at the transcriptional level (Huang et al., 1998) and the latter at the protein activity level (Zhao et al., 1998). Indeed,

we isolated both *crt1* and *sml1* during the initial mutant library screen. In order to ask if *CRT10* belongs to one of the two regulatory pathways, epistasis analysis was performed by creating *crt10*Δ *crt1*Δ and *crt10*Δ *sml1*Δ double mutants and comparing them to the corresponding single mutants with respect to HU resistance. The *crt10*Δ *crt1*Δ double mutant showed the same level of resistance to HU as the *crt1*Δ single mutant (Figure 5-2A), indicating that *CRT10* belongs to the same pathway as *CRT1*. In contrast, the phenotypic effect of *crt10*Δ appears to be additive with *sml1*Δ (Figure 5-2B), suggesting that *CRT10* does not belong to the same regulatory pathway as *SML1*.

### 5.3.4. The transcript level of *RNR* is elevated in *crt10*Δ mutants

Crt1 is an X-box DNA binding protein and represses the transcription of *RNR2*, *RNR3* and *RNR4* through recruitment of the corepressor complex Tup1-Ssn6; deletion of *CRT1* elevated the basal level expression of *RNR3* 25-fold (Huang et al., 1998). The above epistatic analysis predicts that deletion of *CRT10* may result in an elevated *RNR* gene expression as well. The β-gal activities of *RNR3-lacZ* and *RNR2-lacZ* transformants were measured in the wild type and isogenic *crt10*Δ mutants with or without MMS or HU treatment. Indeed, the *RNR3-lacZ* and *RNR2-lacZ* levels were elevated about twofold in *crt10*Δ mutants compared to wild type cells after treatment with DNA damaging agents (Figure 5-3). This result is consistent with a real-time PCR assay of the endogenous *RNR3* transcript (Table 5-1), suggesting that Crt10 functions as a transcriptional repressor to regulate *RNR2* and *RNR3* in budding yeast.

In order to further demonstrate that *CRT10* and *CRT1* belong to the same regulatory pathway, we measured the expression of *RNR3-lacZ* in wild type, *crt1*Δ, *crt10*Δ single and the *crt1*Δ *crt10*Δ double mutants by β-gal assays. As shown in Table

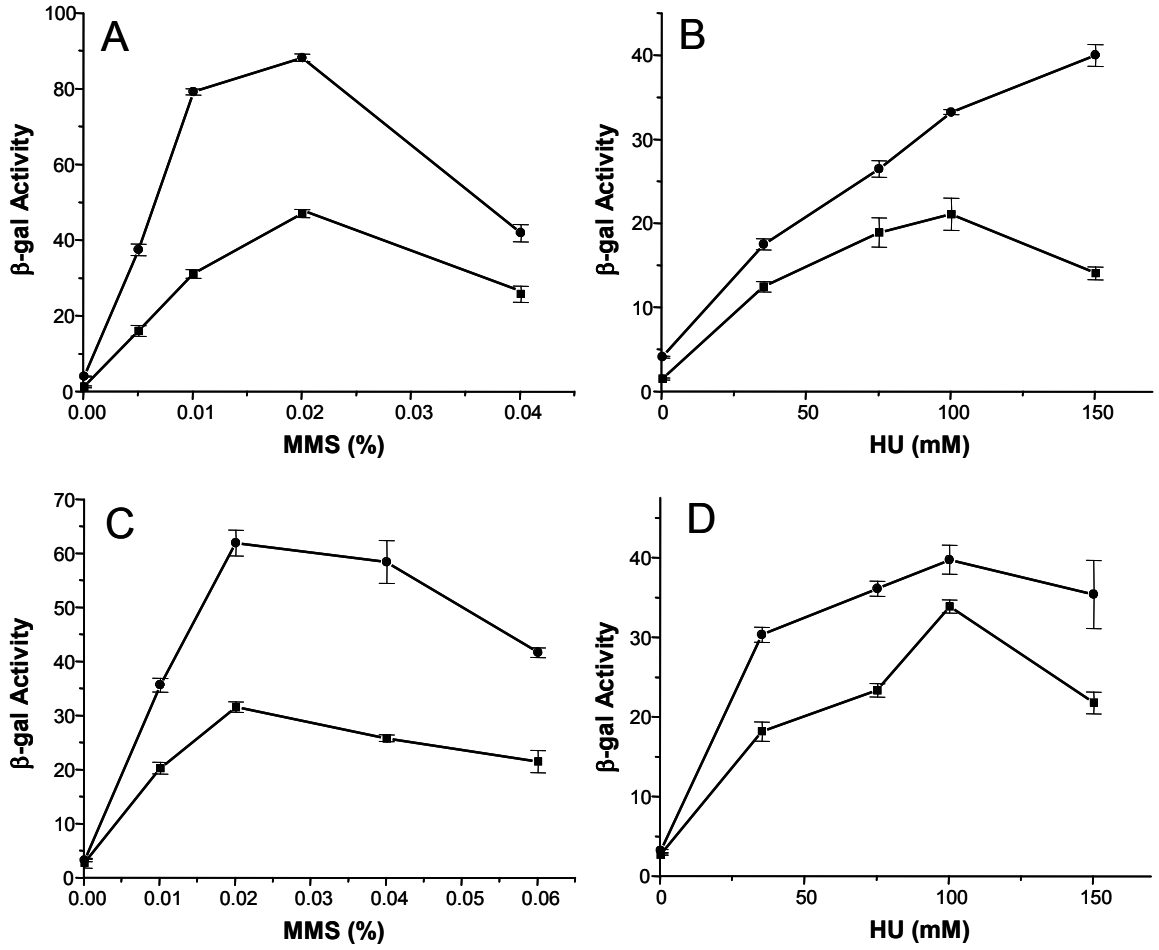



**Figure 5-3. *RNR* gene expression in wild type and *crt10*Δ cells.** *RNR3-lacZ* (A, B) and *RNR2-lacZ* (C, D) expression was monitored after MMS (A, C) and HU (B, D) treatments. β-gal activity was determined as described in Materials and Methods. (■) BY4741 (wild type) and (●) WXY1152 (*crt10*Δ) were transformed with either pZZ2 (pRNR3-lacZ) or pZZ18 (pRNR2-lacZ) and several independent transformants were picked for analysis. The results are the average of at least three independent experiments with standard deviations. β-gal activity is given in Miller units.

**Table 5-1. Relative steady-state transcript level**

| | Relative transcript level[a] | | | |
|---|---|---|---|---|
| Strain | HK578-10D (WT) | | WXY1158 (*crt10*Δ) | |
| Treatment | -HU | +0.2 M HU | -HU | +0.2 M HU |
| *RNR3* | 1 | 9.14 | 2.71 | 26.74 |
| *MAG1* | 1 | 2.32 | 1.27 | 2.19 |
| *TUP1* | 1 | 1.20 | 0.94 | 1.09 |
| *SSN6* | 1 | 0.99 | 1.02 | 0.99 |
| *CRT1* | 1 | 2.06 | 0.38 | 0.50 |

[a] Transcript levels were measured by real-time PCR with total mRNA from cells with or without treatment with 0.2 M HU for 1 h, and normalized to the *ACT1* transcript control. Untreated wild-type cells were used as a reference. Experimental variations due to PCR reaction are negligible.

5-2, the basal level of *RNR3-lacZ* was moderately elevated in the *crt10*Δ mutant and dramatically elevated in the *crt1*Δ mutant. Nevertheless, deletion of *CRT10* does not further enhance *RNR3-lacZ* expression in the *crt1*Δ mutant. The same effect holds true after MMS treatment. These results are consistent with the hypothesis that *CRT10* and *CRT1* function in the same pathway to regulate the transcription of *RNR* genes.

### 5.3.5. *CRT10* functions downstream of *DUN1*

The observation that *crt1* is epistatic to *crt10* with respect to both HU resistance and *RNR* gene activity suggests that Crt1 most likely acts downstream of Crt10. The activity of Crt1 is regulated by its phosphorylation state, and the phosphorylation of Crt1 requires the protein kinase Dun1, although whether Dun1 directly phosphorylates Crt1 remains to be determined (Huang et al., 1998). In order to determine the genetic interaction between *CRT10* and *DUN1*, a *crt10*Δ *dun1*Δ double mutant was created and compared to its corresponding single mutants with respect to HU sensitivity. As seen in Figure 5-4A, whereas deletion of *DUN1* enhances HU sensitivity and deletion of *CRT10* results in HU resistance, cells carrying both deletions display a phenotype indistinguishable from that of *dun1*Δ mutant. Similarly, *dun1* is epistatic to *crt10* with respect to *RNR3* expression, as deletion of *CRT10* did not alter the reduced *RNR3* induction in the *dun1* mutant (Figure 5-4B). These observations indicate that the HU resistance and increased *RNR* expression caused by *CRT10* deletion require functional Dun1.

Crt10 may act either upstream or downstream of Dun1. Dun1 is a multi-functional protein involved in gene regulation (Zhao and Rothstein, 2002) as well as cell cycle checkpoints (Gardner et al., 1999; Maiti et al., 2005). Deletion of *DUN1* not only

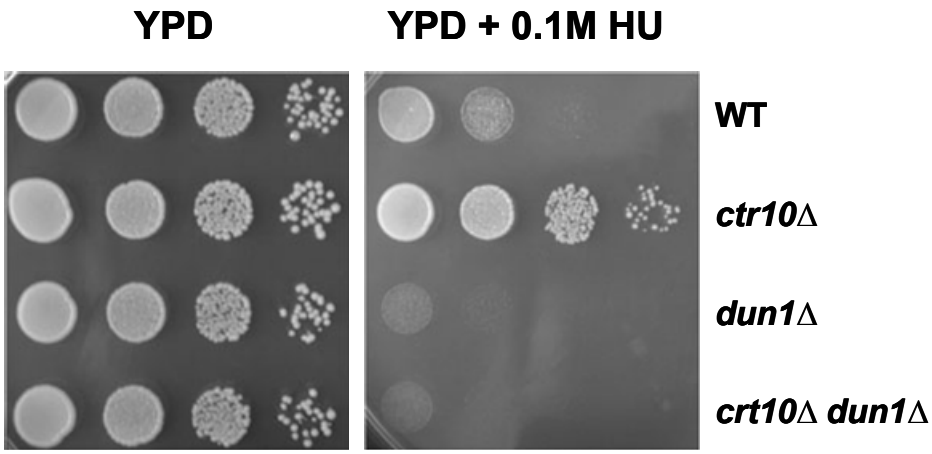
**Table 5-2. β-gal activities of *RNR3-lacZ* in *crt1*Δ and *crt10*Δ mutants**

| Strain[a] | β-Galactosidase Activity (Miller units)[b] | |
|---|---|---|
| | -MMS | +0.02% MMS |
| BY4741 | 1.6 ± 0.12 | 48.9 ± 1.75 |
| WXY1153 (*crt1*Δ) | 72.5 ± 2.72 | 101.3 ± 3.51 |
| WXY1152 (*crt10*Δ) | 4.2 ± 0.32 | 85.5 ± 1.60 |
| WXY1154 (*crt1*Δ *crt10*Δ) | 73.8 ± 3.51 | 100.8 ± 2.74 |

[a] All strains were transformed with pZZ2 (*RNR3-lacZ*).

[b] β-gal activity was measured as described in Materials and Methods. Data represent the averages of at least three independent experiments with standard deviations.

A


YPD
YPD + 0.1M HU
WT
ctr10Δ
dun1Δ
crt10Δ dun1Δ

B

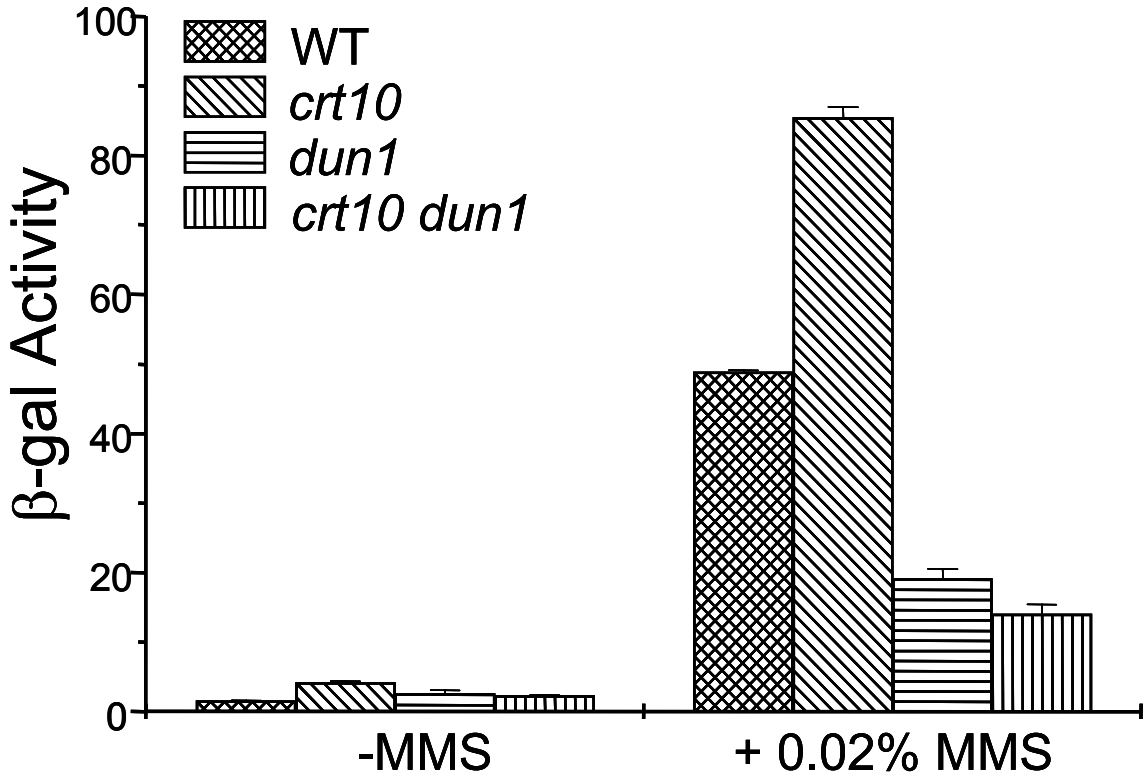

β-gal Activity
100
80
60
40
20
0
WT
crt10
dun1
crt10 dun1
-MMS
+ 0.02% MMS

**Figure 5-4. *DUN1* is epistatic to *CRT10*.** (A) Deletion of *DUN1* abolishes the HU resistance caused by the *crt10* mutation. Ten-fold serial dilutions were spotted on YPD plates and on YPD plates containing 0.1 M HU. Plates were incubated for 3 days at 30$^{o}$C before photographing. Strains used: BY4741 (WT), WXY1152 (*crt10*Δ), WXY1155 (*dun1*Δ), WX1156 (*crt10*Δ *dun1*Δ). (B) The *CRT10* effect on *RNR3* expression is dependent on *DUN1*. *RNR3-lacZ* expression was monitored with or without MMS treatment and expressed in Miller Units. The results are the average of at least three independent experiments with standard deviations.

affects *RNR* gene induction, but also other DNA damage-inducible gene expression (Zhu and Xiao, 2001). We reasoned that if Crt10 acts upstream of Dun1, its inactivation would alter all Dun1-mediated activities. If, as previously observed, Crt10 only affects a subset (i.e., *RNR*) of Dun1-mediated gene expression, inactivation of Crt10 should not affect other gene expression. For example, *MAG1* induction by DNA damage requires Dun1 (Zhu and Xiao, 2001); we found that its expression and induction was not altered by deletion of *CRT10* (Table 5-1), suggesting that indeed Crt10 acts downstream of Dun1 and is specific for *RNR* gene expression.

### 5.3.6. *CRT10* is required for *CRT1* expression and induction

The above genetic analyses fit into a model that Crt10 functions as a positive regulator of Crt1 and/or its co-repressors Tup1-Ssn6. We thus measured the transcript levels of *CRT1*, *TUP1* and *SSN6* with or without HU treatment. As shown in Table 5-1, deletion of *CRT10* does not affect *TUP1* or *SSN6* mRNA regardless of HU treatment, but significantly reduced the basal level as well as HU-induced expression of *CRT1*. Hence, Crt10 appears to serve as a positive regulator of Crt1 at the transcriptional level.

### 5.3.7. Expression of *CRT10* is elevated in response to DNA damage and HU

Many genes involved in DNA metabolism (replication, repair and recombination) are induced after treatment with DNA damaging agents or replication blocking agents. In addition, regulatory genes such as *CRT1* itself are up-regulated in response to DNA damage or HU treatment in a *DUN1*-dependent manner (Huang et al., 1998), indicative of an auto-regulatory circuit. We measured the *CRT10* transcript level in the presence or absence of DNA damaging agents and found that the *CRT10* transcript level is increased

after treatment with MMS, HU and γ-rays (Figure 5-5A). Interestingly, there appear to be two transcripts with slightly different sizes; the treatments induce expression of both transcripts, but the higher molecular weight transcript is induced more dramatically than the lower molecular weight transcript. In order to address whether the transcriptional regulation of *CRT10* is dependent on other regulators in this pathway such as Crt1 and Dun1, we compared the *CRT10* transcript levels in the wild type and mutant backgrounds. The induction of *CRT10* requires *DUN1*, as the *dun1* mutation completely abolished *CRT10* induction, whereas deletion of *CRT1* has no effect on *CRT10* expression (Figure 5-5B).

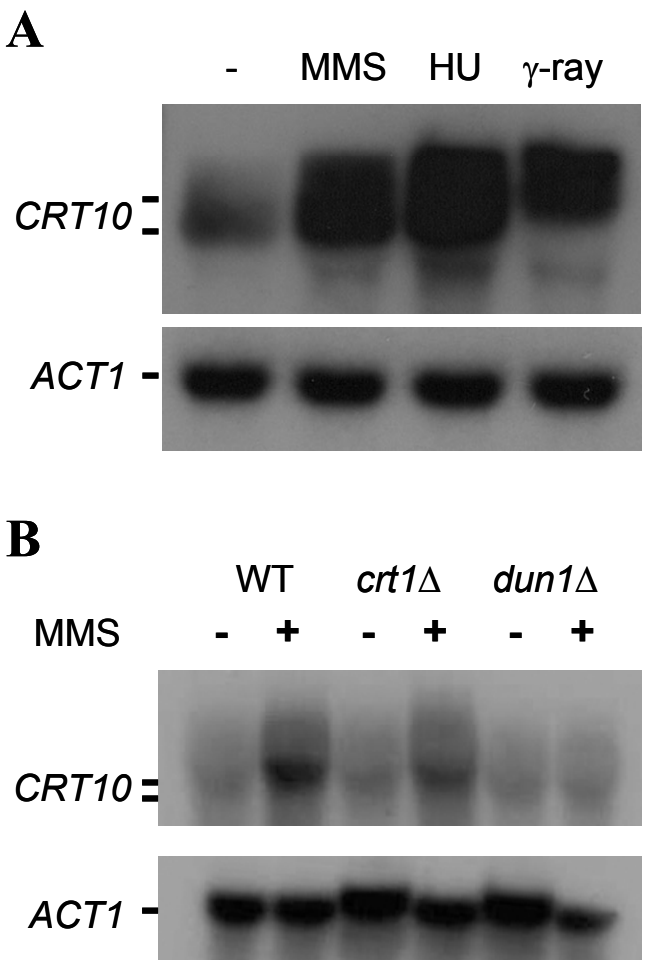



**Figure 5-5.** ***CRT10*** **is a DNA damage-inducible gene. (A)** *CRT10* expression in response to DNA damage and HU treatment. Log-phase wild type HK578-10A cells were either untreated (lane 1) or treated with 0.3% MMS for 2 h (lane 2), 0.2 M HU for 2 h (lane 3), or exposed to 40 krad of γ radiation (lane 4). **(B)** *CRT10* induction is *DUN1*-dependent. Log-phase wild type BY4741 and its derivatives WXY1153 (*crt1*Δ) and WXY1155 (*dun1*Δ) were either untreated (-) or treated with 0.1% MMS for 2 h (+). Northern hybridization was performed as described in Materials and Methods. The membranes were hybridized with *CRT10* (upper panel), stripped and then hybridized with *ACT1* (lower panel) as an internal control. Each lane contains 15 μg of total RNA.

## 5.4. Discussion

We report here the isolation and initial characterization of *CRT10* as a novel yeast gene involved in the transcriptional regulation of *RNR* genes. Rnr catalyzes a rate-limiting step in the production of dNTPs, whose levels are critical to many cellular functions (Zhou and Elledge, 1992). Imbalanced or insufficient dNTP pools lead to enhanced misincorporation, high mutation frequencies and impaired DNA repair (Chabes et al., 2003). Due to its vital importance to cellular physiology, it is not surprising that Rnr is tightly regulated via multiple mechanisms and at different stages. Our results suggest that Crt10 is a newly discovered negative regulator of *RNR* genes and acts at the transcriptional level. First, deletion of *CRT10* results in enhanced cellular resistance to HU, an Rnr inhibitor. Second, deletion of *CRT10* enhances the survival of the *mec1* null mutant, reminiscent of other suppressors that rescue the nonviability of *mec1* and *rad53*, all of which lead to increased Rnr activities (Chabes et al., 1999; Desany et al., 1998; Huang et al., 1998; Zhao et al., 1998). Third, deletion of *CRT10* in wild type cells results in an increased expression of *RNR* genes coding for both large and small Rnr subunits, in the presence and absence of DNA damage, which provides the underlying mechanism of HU resistance. Results obtained from epistasis analyses suggest that Crt10 functions downstream of Dun1 and probably upstream of or together with Crt1 (Figure 5-6). Nevertheless, both *dun1* and *crt1* are epistatic to *crt10*, suggesting that Crt10 is probably a regulatory component in the Dun1-Crt1 signal transduction pathway leading to the control of *RNR* gene expression. Finally, our observation that deletion of *CRT10* reduces *CRT1* expression and abolishes the DNA damage induction of *CRT1* provides direct evidence that *CRT10* functions through positive regulation of *CRT1* expression.

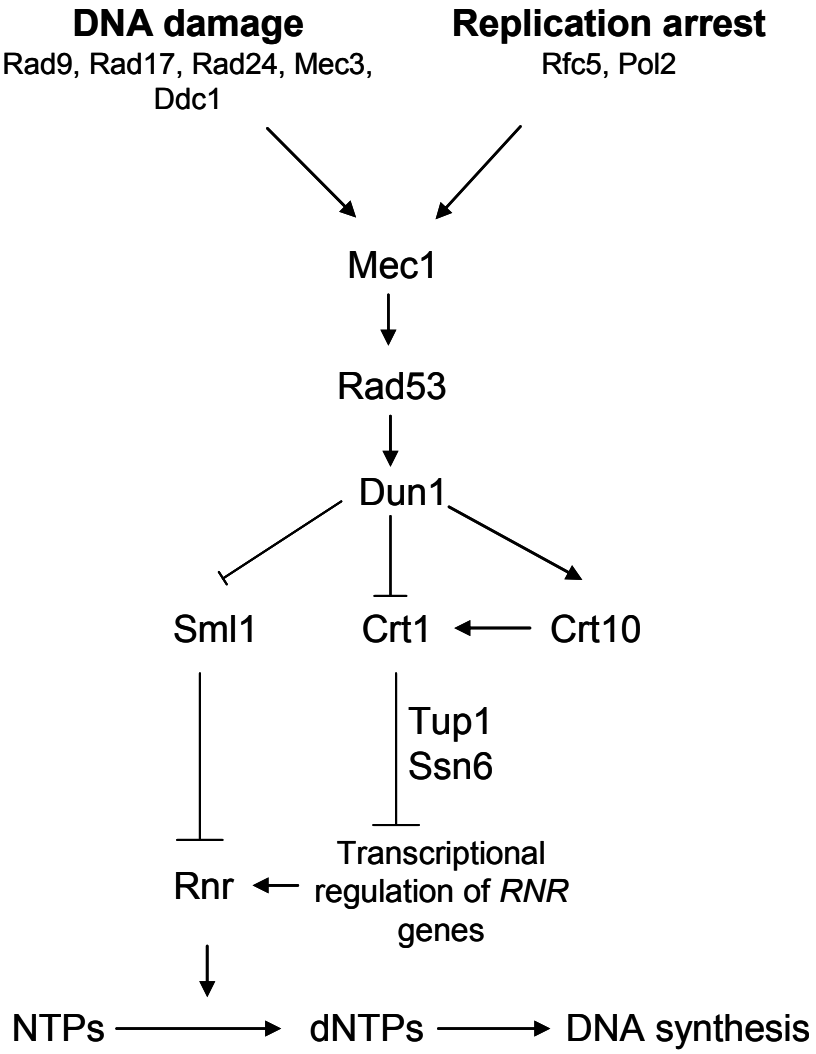



**Figure 5-6. A proposed model for *CRT10* in Rnr regulation.** Note that the *CRT10* functions and regulation are based on its genetic data instead of protein activity. Note that Rnr catalyzes the rate-limiting step in dNTP production.

The physiological significance of *RNR* suppression by *CRT10* is presently unclear. However, one interesting observation through this study is that *CRT10* itself is induced after DNA damage and HU treatment, suggesting that Crt10 plays a critical role in responding to replication blocks. Several pieces of evidence indicate that Crt10 achieves this objective through delicate regulation of the endogenous dNTP pool, as illustrated in Figure 5-6. Firstly, Crt10 acts as a negative regulator to maintain a balanced dNTP pool. In the presence of replication blocks (DNA damage) or with an exhausted dNTP pool (HU treatment to inhibit Rnr activity), all four *RNR* genes are upregulated and Sml1 activity is inhibited, leading to enhanced dNTP production. The increased Crt10 activity may be required to bring Rnr activity back to a normal level once order is restored. In this respect, it is of great interest to notice that the optimal dose required to induce *CRT10* is higher than that required to induce *RNR* genes (Jia et al., 2002), which is consistent with the notion that *CRT10* induction may lag behind that of *RNR* genes. Secondly, like *CRT1* (Huang et al., 1998), the induction of *CRT10* itself depends on *DUN1*, suggesting that Crt10 functions downstream of Dun1 and forms another component of the autoregulatory circuit. However, the effect of *CRT10* deletion on *RNR* gene expression is much less than that of *CRT1* deletion and, unlike *CRT1*, the *CRT10* promoter does not contain the X-box sequence recognized by Crt1 (Huang et al., 1998). This is not unprecedented since DNA damage induction of several other genes also requires *DUN1* in the absence of the X-box sequence (Zhu and Xiao, 2001). Finally, in addition to its roles in modulating and maintaining an optimal dNTP pool under stress conditions, *CRT10* appears to be required for optimal growth in the absence of exogenous DNA damage/replication blocks, since a recent genetic footprinting study (Dunn et al., 2004) showed that deletion of *CRT10* causes an apparently severe growth defect in rich medium

after 20 generations, in minimal medium, as well as medium containing NaCl. These observations imply that vigorous modulation of the endogenous dNTP pool by *CRT10* is critical to achieve optimal cell growth, possibly by maintaining proper DNA synthesis and cell division. Alternatively, *CRT10* may play roles in optimizing cell growth by a mechanism other than affecting Rnr activity.

Despite the strong genetic evidence that Crt10 is involved in the transcriptional regulation of *RNR* genes and *CRT1*, its biochemical activity remains obscure. The leucine repeats and a single WD motif suggest that Crt10 may interact with other protein(s), although to date no such proteins have been identified through systematic studies. It does not contain a domain/motif indicative of its catalytic function. However, the protein is apparently conserved and widespread within unicellular lower eukaryotes. Future investigations will attempt to uncover biological and biochemical functions of Crt10.

# CHAPTER SIX

## SUMMARY

In summary, this study elucidates the DNA damage induced transcription regulation network in budding yeast from both the general DNA damage sensor and the detailed regulatory mechanisms of specific genes.

In general, a straightforward DNA damage induction pathway model comprises damage sensor, signal transducer and downstream effector. It has been hypothesized that DNA repair proteins recognize DNA lesions in the early stage of DNA repair so that they may also regulate the transcription in response to DNA damage by sensing lesions or replication blocks. By analyzing the expression of two co-ordinately regulated damage inducible genes *MAG1* and *DDI1* in different DNA repair defective mutants, it was found that among major DNA repair pathways, only mutations in *RAD6* and *RAD18* of the PRR pathway affected the damage induction of *MAG1* and *DDI1*. Furthermore, the microarray analysis showed that *rad6* and *rad18* mutations appear to variably affect the expression of 379 DNA damage inducible genes. Rad6 and Rad18 form a heterodimer *in vivo*, and Rad18 possesses the ability to bind single-stranded DNA and displays single-stranded DNA dependent ATPase activity. All these make the Rad6-Rad18 complex a strong candidate to activate DNA damage induction by sensing damage signals.

In budding yeast, the protein kinase Rad53 is one of the transducers in the DNA damage induction pathway, which has prominent effect on DNA damage induced transcription of many genes. Rad53 can be activated by phosphorylation in response to

DNA damage, and the phosphorylation of Rad53 is regulated by two parallel pathways represented by *RAD24* and *SGS1*. This study showed that Rad53 phosphorylation in response to MMS treatment is dramatically reduced in the *rad18Δ sgs1Δ* double mutant but not in the *rad18Δ rad24Δ* double mutants. It suggests that the Rad6-Rad18 complex functions in the Rad24 checkpoint pathway parallel to the Sgs1 pathway to induce Rad53 phosphorylation. This conclusion is also supported by the results obtained from gene expression assays for two DNA damage inducible genes *MAG1* and *RNR3*: the effect of *rad18* and *rad24* mutations on the induction of both *MAG1* and *RNR3* is epistatic, whereas that of *rad18* or *rad24* is additive to the *sgs1* mutation.

Moreover, the mechanism by which the Rad6-Rad18 complex transfers the DNA damage signal downstream has been investigated. Rad6-Rad18 is a ubiquitination complex, and its only activity reported to date is to mono-ubiquitinate the *POL30* product PCNA at the Lys164 residue and initiate the PRR pathway. Therefore, it has been examined whether Rad6-Rad18 also initiates DNA damage induction through ubiquitinating PCNA. However, my study reveals that neither the ubiquitination nor the sumoylation of PCNA is involved in DNA damage induction. In budding yeast, the 9-1-1 complex is a PCNA-like clamp involved in DNA damage responses. Combined with the fact that Rad24 functions as a clamp loader for the 9-1-1 complex in DNA damage responses, it is likely that Rad6-Rad18 initiates DNA damage induction through ubiquitinating a subunit of the 9-1-1 complex. In a yeast two-hybrid analysis, only the subunit Rad17 in the 9-1-1 complex showed a physical interaction with Rad18. Further studies revealed that Rad17 can be ubiquitinated and the ubiquitination is both Rad6-Rad18 and DNA-damage dependent. The results from β-gal assays suggest that the putative ubiquitination site lysine 197 in Rad17 is required to mediate the DNA damage

induction by Rad6-Rad18. It is conceivable that the Rad6-Rad18 complex passes down the DNA damage signal to the transducer Rad53 through ubiquitination of the Rad17 subunit of the in 9-1-1 complex.

Among all components in the DNA damage induction pathway, the downstream effectors are mostly uncharacterized. Unlike the upstream components, such as sensors and transducers, which regulate the expression of many genes, each downstream effector seems to only control a particular set of inducible genes in the DNA damage response. These downstream effectors interact with unique *cis*-acting elements to accomplish the optimized regulation by either derepression or direct activation. In this study, we also investigated some *cis*-acting elements and *trans*-acting factors for specific DNA damage inducible genes.

During the microarray assay, we found that the transcription of two genes *YFL061w* and *YNL335w* were induced up to 100-fold by MMS treatment. These two genes are identical genes with similar promoter region. We designated them *DDI2* and *DDI3* respectively. Northern hybridization and β-gal assay further confirm that MMS treatment can highly induce the transcription of *DDI2*/*DDI3*. Interestingly, only the $SN_2$ alkylating agents, such as MMS and DMS, highly induced the transcription of *DDI2*/*DDI3*. Other DNA damaging agents like $SN_1$ alkylating agents, HU and γ-ray hardly induced the transcription. In order to elucidate the mechanisms of induction, the *cis*-acting elements in the promoter region have been mapped. Deletion of the region extending from -358 to -229 in the *DDI2* promoter area decreased both basal-level expression and the induction by MMS, suggesting that a UAS is located in this region. This UAS also functioned in the context of a heterologous promoter to result in MMS induced expression of the *CYC1-lacZ* reporter gene. Meanwhile, a URS was identified in

the region between -709 and -543. According to our data, it is likely that UAS and URS regulate the MMS induced transcription of *DDI2* cooperatively.

In budding yeast, *RNR* genes are well-documented DNA damage inducible genes. The DNA damage induction of this gene family is regulated by the *trans*-acting effectors binding to their promoter region, such as repressor Crt1. In this study, I cloned a novel gene *CRT10*, which encodes a negative regulator of the ribonucleotide reductase activity. Deletion of *CRT10* increased basal level and DNA damage-induced expression of *RNR* gene, and the Crt10 function appears to be achieved by positive regulation of the *CRT1* transcript level. Furthermore, *dun1* is epistatic to *crt10* with respect to both HU sensitivity and *RNR* gene regulation. Interestingly, the expression of *CRT10* itself is induced by DNA damaging agents and this induction requires *DUN1*. Taken together, it seems that *CRT10* is a component of the regulatory circuit controlling the Rnr activity.

Overall, this thesis elucidates that transcription regulation of a number of yeast DNA damage-inducible genes occurs through a signal transduction pathway mediated by the Rad6-Rad18 complex, and this complex might function as a DNA damage sensor in the signal transduction pathway. In addition, this study investigated the transcriptional regulation of specific damage-inducible genes and identified novel *cis*-acting elements and *trans*-acting factors contributing to the DNA damage induction cascade.

# CHAPTER SEVEN

# CONCLUSIONS AND FUTURE DIRECTIONS

## 6.1. CONCLUSIONS

- The Rad6-Rad18 complex is involved in the transcriptional regulation in response to DNA damage.
- It appears that the DNA damage signal recognized by Rad6-Rad18 is relayed to the cascade of cell cycle checkpoints.
- The Rad17 subunit of the 9-1-1 complex is subject to Rad6/Rad18- and DNA damage-dependent mono-ubiquitination.
- The Rad6-Rad18 complex is likely to mediate a eukaryotic SOS response by coordinating translesion synthesis, error-free bypass, homologous recombination, cell cycle checkpoints as well as transcriptional regulation.
- *DDI2* and *DDI3* are two identical genes whose transcription can be highly induced by MMS treatment.
- The high level induction of *DDI2/DDI3* is specific for the treatment by $SN_2$ alkylating agents.
- The promoter region $UAS_{DDI2}$ (-358 to –229) is responsible for the high level induction of *DDI2/DDI3*.
- *CRT10* encodes a negative regulator of ribonucleotide reductase activity, and deletion of *CRT10* increases basal level and DNA damage-induced expression of *RNR* genes.

- The Crt10 function appears to be achieved by positive regulation of the *CRT1* transcript level.
- It seems that *CRT10* is a component of the regulatory circuit controlling Rnr activity.

## 6.2. FUTURE DIRECTIONS

A number of future considerations were brought up in each study of this thesis. The future directions of immediate interest are discussed below:

- **To obtain direct evidence to show that the Lys197 residue in Rad17 is the ubiquitination site for the Rad6-Rad18 complex**

  In previous studies, we hypothesized that Lys197 in Rad17 is the putative ubiquitination site according to the strong conservation of flanking sequence between Rad17-Lys197 and Pol30-Lys164. We further demonstrated that the effects of the *rad17-K197R* mutation on *MAG1* and *RNR3* induction are indistinguishable from that of *rad17*Δ cells. However, direct evidence is still missing. Further experimental approaches include the creation of a chromosomally Myc-tagged *rad17-K197R* strain and monitoring its ubiquitination by western blot analysis and Co-IP. Moreover, the effect of this point mutation along with the *rad17* null mutation on Rad53 phosphorylation will also be examined. These tests will provide direct evidence that Lys197 is the ubiquitination site for the Rad6-Rad18 complex.

- **To investigate the role of *RAD18/RAD6* in DNA damage checkpoint**

  Data presented in Chapter Three imply that Rad18/Rad6 could function in the DNA damage checkpoint. As mentioned in the Discussion section of Chapter

Three, the previous checkpoint studies normally used UV and ionizing radiation as the main damage sources of DNA damage, but *RAD6* and *RAD18* have not been implicated in the damage checkpoint pathway in these studies. In future studies, we will use MMS or HU to induce DNA damage and cause replication stress, which are likely to trigger the intra-S phase checkpoint. Cells of a wild type and its isogenic deletion mutant will be synchronized in early $G_1$ by α-factor and immediately released in media containing appropriate concentrations of MMS or HU. Cell cycle progression can be monitored as a function of time by flow-cytometric analysis of DNA content. Since the dual functions of Rad6-Rad18 in PRR and damage checkpoint may undermine detection of its checkpoint function, we will compare the cell cycle progression between *pol30-K164R* mutants and *pol30-K164R rad18*Δ double mutants. If *RAD18* is involved in the DNA damage checkpoint, compared with the single mutant, the double mutant should show faster cell cycle progression after treatment. These studies could help us to assess whether *RAD6/RAD18* function in the DNA damage checkpoint pathway.

- **To identify the *trans*-acting regulatory factors of *DDI2/DDI3***

Results presented in Chapter Four show that the transcription of *DDI2/DDI3* can be highly induced by MMS treatment. The promoter region of *DDI2/DDI3* has been analyzed and several *cis*-acting elements were identified. However, the *trans*-acting regulatory factors functioning in the *DDI2/DDI3* promoter region remain unclear. A new technique called synthetic genetic array (SGA) provides us with a powerful tool to identify these regulatory factors at the total genome level. A query strain which contains a chromosomally integrated *DDI2-lacZ* will be constructed. Since the expression level of *DDI2/DDI3* is very low without treatment and increases

dramatically after MMS treatment, the query strain should show no color on X-gal plates and show blue color on X-gal + MMS plates. The query strain will be crossed to an array of approximately 5000 viable yeast single deletion mutants. Through a series of replica-pinning procedures, the strain containing both single deletion and *DDI2-lacZ* will be selected and scored for blue colour display on X-gal + MMS plates. Applying SGA analysis may enable us to identify all positive or negative *trans*-acting regulatory factors in the yeast genome. It will shed light on the understanding of molecular mechanism by which *DDI2/DDI3* is regulated in response to MMS treatment. A similar strategy can also be applied to study other DNA damage-inducible genes such as *RNR3* and *MAG1*.

- **To investigate how Crt10 contributes to the transcriptional regulation of Crt1.**

  In Chapter Five, we demonstrated that Crt10 is a positive regulator for the transcription of *CRT1*. So far, its biochemical activity remains obscure. Future studies are proposed to first determine whether Crt10 physically interacts with the *CRT1* promoter region with a super-shift assay and chromatin immunoprecipitation. As mentioned in the Discussion section of Chapter Five, the existence of leucine repeats and a single WD motif in Crt10 suggest that it may interact with other protein(s). A yeast two-hybrid screen can be carried out to identify the protein(s) interacting with Crt10. Recently, Crt10 has been suggested to physically interact with Cdc34 (Krogan et al., 2006) and Rtt101 (Collins et al., 2007) in global Affinity Capture-MS experiments. Both proteins are components of the ubiquitin conjugating complexes involved in cell cycle regulation. Thus, it remains possible that Crt10 is modified by ubiquitination in response to cell cycle stress. Since the transcription of *RNR* genes is cell cycle regulated, it would be interesting to verify whether Crt10 can

be modified by these ubiquitin conjugating complexes and to determine its functional alteration after the modification.

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# APPENDIX A

## Microarray Raw Data

***Saccharomyces cerevisiae* genes whose DNA damage induction is affected by both *rad6*Δ and *rad18*Δ.**

| ID | Gene | Wt | rad18 | rad6 |
|---|---|---|---|---|
| YAL043C | PTA1 | 2.117 | 0.837 | 0.923 |
| YAR009C | | 2.538 | 0.957 | 0.842 |
| YBR005W | RCR1 | 2.049 | 0.664 | 0.796 |
| YBR008C | FLR1 | 7.919 | 4.452 | 2.999 |
| YBR053C | | 2.176 | 0.805 | 1.121 |
| YBR056W | ECM2 | 2.311 | 1.266 | 1.07 |
| YBR068C | BAP2 | 2.808 | 0.809 | 1.161 |
| YBR072W | HSP26 | 5.149 | 1.999 | 1.355 |
| YBR126C | TPS1 | 2.064 | 0.725 | 1.255 |
| YBR149W | ARA1 | 2.364 | 0.968 | 1.123 |
| YBR169C | SSE2 | 3.246 | 1.467 | 1.598 |
| YBR170C | NPL4 | 2.06 | 1.066 | 1.274 |
| YBR256C | RIB5 | 5.531 | 1.998 | 2.291 |
| YCL026C | | 2.047 | 1.193 | 1.262 |
| YCL030C | HIS4 | 3.7 | 0.337 | 0.441 |
| YCL032W | STE50 | 2.12 | 0.867 | 0.951 |
| YCL034W | LSB5 | 3.188 | 0.876 | 1.382 |
| YCL038C | AUT4 | 2.49 | 0.886 | 0.876 |
| YCL039W | GID7 | 3.435 | 1.318 | 1.688 |
| YCL040W | GLK1 | 2.792 | 0.85 | 0.797 |
| YCL049C | | 2.037 | 0.5 | 1.052 |
| YCLX04W | | 3.697 | 1.088 | 1.704 |
| YCLX07W | | 4.178 | 1.183 | 0.797 |
| YCLX08C | FRM2 | 6.434 | 1.122 | 1.906 |
| YCR011C | ADP1 | 2.258 | 0.789 | 1.127 |
| YCR030C | SYP1 | 2.162 | 0.711 | 0.965 |
| YCR036W | RBK1 | 2.22 | 1.257 | 0.973 |
| YCR107W | AAD3 | 7.005 | 2.536 | 1.562 |
| YCRX21C | | 2.612 | 1.217 | 1.122 |
| YDL019C | OSH2 | 2.318 | 0.627 | 1.079 |
| YDL021W | GPM2 | 3.424 | 0.751 | 1.164 |
| YDL025C | | 5.12 | 1.769 | 1.279 |
| YDL048C | STP4 | 4.04 | 0.649 | 0.689 |
| YDL066W | IDP1 | 2.522 | 0.79 | 0.698 |
| YDL070W | BDF2 | 3.149 | 1.232 | 0.954 |
| YDL122W | UBP1 | 2.119 | 0.969 | 0.628 |
| YDL123W | SNA4 | 3.188 | 1.243 | 1.162 |
| YDL126C | CDC48 | 2.914 | 1.169 | 1.003 |
| YDL132W | CDC53 | 3.36 | 1.6 | 0.944 |
| YDL196W | GYP7 | 2.618 | 0.093 | 0.425 |
| YDL234C | GYP7 | 3.66 | 1.472 | 1.136 |

| | | | | |
|---|---|---|---|---|
| YDR003W | RCR2 | 4.189 | 1.858 | 1.325 |
| YDR035W | ARO3 | 3.322 | 1.007 | 0.845 |
| YDR036C | EHD3 | 2.076 | 0.793 | 0.851 |
| YDR074W | TPS2 | 2.533 | 0.568 | 0.578 |
| YDR085C | AFR1 | 2.87 | 0.805 | 0.817 |
| YDR117C | TMA64 | 2.236 | 0.961 | 1.463 |
| YDR124W | | 4.334 | 1.103 | 1.147 |
| YDR137W | RGP1 | 2.408 | 1.397 | 1.22 |
| YDR168W | CDC37 | 2.283 | 1.347 | 1.343 |
| YDR171W | HSP42 | 25.116 | 5.488 | 4.674 |
| YDR175C | RSM24 | 2.841 | 0.931 | 1.168 |
| YDR202C | RAV2 | 2.167 | 0.822 | 1.108 |
| YDR204W | COQ4 | 2.386 | 0.62 | 1.137 |
| YDR254W | CHL4 | 2.447 | 1.747 | 1.621 |
| YDR257C | RMS1 | 3.536 | 1.215 | 0.666 |
| YDR258C | HSP78 | 6.022 | 2.377 | 1.564 |
| YDR259C | YAP6 | 2.349 | 0.536 | 0.983 |
| YDR264C | AKR1 | 2.542 | 1.054 | 1.072 |
| YDR293C | SSD1 | 2.839 | 0.689 | 0.515 |
| YDR380W | ARO10 | 6.171 | 2.626 | 2.035 |
| YDR389W | SAC7 | 2.167 | 1.045 | 0.617 |
| YDR391C | | 2.567 | 1.056 | 1.341 |
| YDR394W | RPT3 | 3.987 | 2.581 | 2.222 |
| YDR443C | SSN2 | 25.446 | 2.154 | 5.611 |
| YDR516C | | 2.078 | 0.552 | 1.011 |
| YDR531W | EMI2 | 2.38 | 0.886 | 1.045 |
| YEL060C | PRB1 | 2.75 | 1.447 | 0.979 |
| YER035W | EDC2 | 2.125 | 0.863 | 0.911 |
| YER053C | PIC2 | 2.056 | 0.788 | 1.018 |
| YER054C | GIP2 | 2.173 | 1.084 | 0.899 |
| YER073W | ALD5 | 2.676 | 0.841 | 0.78 |
| YER087C-A | SBH1 | 2.736 | 0.692 | 1.268 |
| YER090W | TRP2 | 2.452 | 0.87 | 0.934 |
| YER103W | SSA4 | 5.975 | 1.132 | 0.711 |
| YER124C | DSE1 | 2.4 | 0.72 | 0.954 |
| YER142C | MAG1 | 2.176 | 1.372 | 1.299 |
| YER143W | DDI1 | 5.644 | 2.224 | 2.624 |
| YER150W | SPI1 | 4.999 | 1.687 | 1.718 |
| YER169W | RPH1 | 2.81 | 1.032 | 0.959 |
| YER175C | TMT1 | 13.247 | 0.656 | 1.39 |
| YER176W | ECM32 | 2.689 | 1.107 | 0.888 |
| YFL014W | HSP12 | 2.638 | 1.101 | 1.188 |
| YFL049W | SWP82 | 3.273 | 0.549 | 0.885 |
| YFL056C | AAD6 | 8.438 | 1.796 | 0.906 |
| YFL057C | AAD16 | 11.06 | 3.636 | 3.036 |
| YFL061W | DDI2 | 107.884 | 18.854 | 17.908 |
| YFR003C | YPL1 | 2.21 | 1.179 | 1.559 |
| YFR005C | SAD1 | 2.243 | 1.267 | 0.871 |
| YFR040W | SAP155 | 2.823 | 1.426 | 0.992 |
| YFR053C | HXK1 | 4.056 | 1.309 | 1.002 |
| YFR055W | IRC7 | 2.493 | 0.439 | 0.715 |
| YGL006W | PMC1 | 2.268 | 0.434 | 0.871 |
| YGL037C | PNC1 | 5.639 | 3.011 | 3.056 |
| YGL059W | | 2.197 | 1.308 | 1.308 |
| YGL117W | | 19.279 | 1.218 | 1.909 |
| YGL121C | GPG1 | 2.046 | 0.973 | 1.328 |
| YGL160W | | 2.025 | 1.012 | 1.284 |
| YGL180W | APG1 | 4.14 | 1.36 | 1.259 |

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|---|---|---|---|---|
| YGL181W | GTS1 | 3.417 | 2.429 | 1.91 |
| YGL184C | STR3 | 4.923 | 1.048 | 1.438 |
| YGL185C | | 2.006 | 0.636 | 1.041 |
| YGL218W | | 3.001 | 0.717 | 0.679 |
| YGL224C | SDT1 | 2.474 | 1.619 | 1.263 |
| YGL228W | SHE10 | 3.439 | 1.843 | 1.367 |
| YGL229C | SAP4 | 2.833 | 0.981 | 1.09 |
| YGL236C | MTO1 | 2.058 | 0.49 | 0.87 |
| YGL248W | PDE1 | 5.496 | 1.73 | 1.064 |
| YGL250W | RMR1 | 2.161 | 1.359 | 1.694 |
| YGL254W | FZF1 | 2.352 | 0.946 | 0.872 |
| YGR010W | NMA2 | 2.171 | 0.911 | 1.137 |
| YGR023W | MTL1 | 2.379 | 1.135 | 1.476 |
| YGR130C | | 5.051 | 1.575 | 1.424 |
| YGR138C | TPO2 | 4.094 | 1.056 | 0.823 |
| YGR142W | BTN2 | 47.226 | 6.946 | 7.3 |
| YGR154C | GTO1 | 2.033 | 0.728 | 1.029 |
| YGR186W | TFG1 | 2.438 | 1.231 | 0.724 |
| YGR189C | CRH1 | 2.393 | 0.821 | 1.152 |
| YGR194C | XKS1 | 2.222 | 0.418 | 0.67 |
| YGR196C | FYV8 | 2.285 | 1.228 | 1.089 |
| YGR211W | ZPR1 | 5.224 | 1.551 | 1.055 |
| YGR213C | RTA1 | 3.548 | 0.819 | 0.908 |
| YGR237C | | 4.086 | 1.639 | 2.116 |
| YGR248W | SOL4 | 2.124 | 1.027 | 1.217 |
| YGR250C | | 4.482 | 0.958 | 1.159 |
| YHL002W | HSE1 | 4.118 | 1.753 | 2.548 |
| YHL021C | FMP12 | 5.909 | 1.23 | 1.028 |
| YHL027W | RIM101 | 2.728 | 0.885 | 0.681 |
| YHR018C | ARG4 | 10.62 | 2.236 | 1.696 |
| YHR022C | | 3.52 | 0.478 | 0.828 |
| YHR027C | RPN1 | 3.374 | 1.155 | 1.335 |
| YHR028C | DAP2 | 2.497 | 1.042 | 1.318 |
| YHR030C | SLT2 | 6.42 | 0.534 | 0.786 |
| YHR071W | PCL5 | 3.469 | 1.039 | 1.109 |
| YHR082C | KSP1 | 2.227 | 0.651 | 0.935 |
| YHR087W | | 5.216 | 1.577 | 2.063 |
| YHR097C | | 2.558 | 1.267 | 1.276 |
| YHR104W | GRE3 | 2.078 | 1.223 | 1.36 |
| YHR107C | CDC12 | 4.193 | 0.654 | 1.178 |
| YHR137W | ARO9 | 3.369 | 1.882 | 1.616 |
| YHR195W | NVJ1 | 2.24 | 0.912 | 1.031 |
| YHR199C | FMP34 | 2.472 | 1.686 | 1.255 |
| YHR209W | CRG1 | 5.739 | 1.61 | 0.836 |
| YIL001W | | 2.678 | 1.194 | 1.444 |
| YIL002C | INP51 | 2.197 | 0.767 | 1.237 |
| YIL005W | EPS1 | 2.277 | 1.36 | 1.172 |
| YIL044C | AGE2 | 2.139 | 1.219 | 1.001 |
| YIL055C | | 3.107 | 1.577 | 1.232 |
| YIL056W | VHR1 | 3.316 | 1.732 | 1.241 |
| YIL066C | RNR3 | 3.264 | 0.908 | 1.248 |
| YIL082W | | 2.634 | 0.969 | 1.006 |
| YIL101C | XBP1 | 2.615 | 1.873 | 1.711 |
| YIL107C | PFK26 | 3.271 | 1.476 | 1.228 |
| YIL108W | | 2.333 | 0.606 | 1.097 |
| YIL113W | SDP1 | 2.778 | 0.464 | 1.055 |
| YIL116W | HIS5 | 6.353 | 1.239 | 0.888 |
| YIL117C | PRM5 | 5.592 | 1.669 | 0.872 |

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|---|---|---|---|---|
| YIL136W | OM45 | 3.304 | 1.36 | 1.058 |
| YIL137C | TMA108 | 2.147 | 1 | 1.025 |
| YIL151C | | 2.281 | 1.292 | 1.048 |
| YIL164C | NIT1 | 4.624 | 0.815 | 0.874 |
| YIL165C | | 5.88 | 1.655 | 2.143 |
| YIL168W | SDL1 | 2.231 | 0.979 | 1.206 |
| YIL170W | HXT12 | 2.401 | 0.893 | 1.101 |
| YIR002C | MPH1 | 2.073 | 1.06 | 1.005 |
| YIR003W | | 2.332 | 0.871 | 1.231 |
| YIR016W | | 2.376 | 1.372 | 1.686 |
| YIR039C | YPS6 | 3.979 | 0.887 | 1.173 |
| YIR044C | | 3.488 | 1.162 | 0.929 |
| YJL017W | | 2.406 | 0.729 | 0.9 |
| YJL023C | PET130 | 3.321 | 1.841 | 2.077 |
| YJL034W | KAR2 | 2.731 | 1.576 | 1.631 |
| YJL053W | PEP8 | 2.728 | 1.183 | 0.908 |
| YJL057C | IKS1 | 2.1 | 1.615 | 1.356 |
| YJL060W | BNA3 | 2.132 | 1.415 | 1.295 |
| YJL084C | ALY2 | 2.33 | 1.57 | 1.433 |
| YJL085W | EXO70 | 2.857 | 1.312 | 1.391 |
| YJL094C | KHA1 | 2.661 | 1.017 | 1.382 |
| YJL099W | CHS6 | 2.145 | 1.18 | 1.093 |
| YJL111W | CCT7 | 2.266 | 1.068 | 1.084 |
| YJL139C | YUR1 | 2.178 | 1.065 | 1.015 |
| YJL141C | YAK1 | 3.008 | 1.765 | 1.353 |
| YJL144W | | 20.719 | 2.639 | 2.019 |
| YJL155C | FBP26 | 4.661 | 1.968 | 1.36 |
| YJL159W | HSP150 | 2.616 | 0.928 | 0.522 |
| YJL160C | | 4.018 | 0.415 | 0.892 |
| YJL164C | TPK1 | 4.73 | 1.737 | 1.302 |
| YJL165C | HAL5 | 2.569 | 1.074 | 0.942 |
| YJL213W | | 4.65 | 0.628 | 1.244 |
| YJL219W | HXT9 | 2.212 | 0.686 | 1.231 |
| YJR025C | BNA1 | 2.597 | 0.925 | 0.951 |
| YJR035W | RAD26 | 2.284 | 0.867 | 1.188 |
| YJR045C | SSC1 | 3.196 | 0.622 | 0.934 |
| YJR046W | TAH11 | 4.197 | 1.708 | 1.168 |
| YJR052W | RAD7 | 2.862 | 1.189 | 0.933 |
| YJR059W | PTK2 | 2.947 | 0.651 | 1.082 |
| YJR062C | NTA1 | 2.938 | 0.851 | 0.897 |
| YJR096W | | 3.727 | 2.03 | 1.86 |
| YJR109C | CPA2 | 9.036 | 0.567 | 1.463 |
| YJR110W | YMR1 | 3.506 | 0.801 | 1.253 |
| YJR130C | STR2 | 10.032 | 1.895 | 1.427 |
| YJR152W | DAL5 | 2.109 | 0.928 | 0.731 |
| YKL010C | UFD4 | 2.31 | 0.87 | 1.345 |
| YKL015W | PUT3 | 2.597 | 0.972 | 1.308 |
| YKL022C | CDC16 | 2.728 | 1.601 | 1.603 |
| YKL023W | | 3.633 | 1.441 | 1.107 |
| YKL025C | PAN3 | 3.438 | 1.267 | 1.13 |
| YKL035W | UGP1 | 2.818 | 0.828 | 0.512 |
| YKL051W | SFK1 | 2.023 | 1.112 | 0.79 |
| YKL052C | ASK1 | 2.157 | 1.17 | 0.974 |
| YKL062W | MSN4 | 2.065 | 1.037 | 0.719 |
| YKL064W | MNR2 | 2.498 | 0.619 | 0.985 |
| YKL070W | | 6.789 | 1.876 | 2.294 |
| YKL071W | | 49.892 | 7.362 | 13.157 |
| YKL088W | | 2.106 | 0.591 | 0.983 |

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|---|---|---|---|---|
| YKL091C | | 2.369 | 1.456 | 1.307 |
| YKL103C | LAP4 | 8.236 | 2.793 | 1.763 |
| YKL104C | GFA1 | 3.027 | 0.542 | 0.814 |
| YKL105C | | 2.154 | 0.843 | 1.16 |
| YKL107W | | 2.565 | 1.335 | 1.003 |
| YKL133C | | 2.944 | 1.149 | 1.292 |
| YKL146W | AVT3 | 3.689 | 1.856 | 1.602 |
| YKL148C | SDH1 | 2.64 | 0.65 | 0.757 |
| YKL151C | | 3.455 | 1.663 | 1.036 |
| YKL163W | PIR3 | 4.461 | 1.066 | 0.465 |
| YKL170W | MRPL38 | 3.467 | 1.052 | 0.994 |
| YKL193C | SDS22 | 2.298 | 1.254 | 1.273 |
| YKL211C | TRP3 | 4.242 | 1.014 | 0.784 |
| YKL213C | DOA1 | 2.783 | 1.702 | 1.495 |
| YKL222C | | 2.065 | 0.869 | 0.804 |
| YKR002W | PAP1 | 3.533 | 1.902 | 1.572 |
| YKR003W | OSH6 | 2.129 | 1.19 | 1.361 |
| YKR008W | RSC4 | 3.49 | 1.697 | 0.981 |
| YKR011C | TOS5 | 7.187 | 3.973 | 2.578 |
| YKR015C | | 2.013 | 1.003 | 1.06 |
| YKR061W | KTR2 | 3.074 | 0.951 | 1.069 |
| YKR069W | MET1 | 2.808 | 0.513 | 0.571 |
| YKR104W | | 3.369 | 1.747 | 1.525 |
| YLL019C | KNS1 | 2.169 | 0.814 | 0.949 |
| YLL026W | HSP104 | 10.415 | 2.028 | 2.752 |
| YLL028W | TPO1 | 2.057 | 0.569 | 0.816 |
| YLL056C | | 3.321 | 2.619 | 2.26 |
| YLL060C | GTT2 | 10.206 | 4.547 | 2.416 |
| YLR001C | | 3.201 | 1.32 | 1.309 |
| YLR023C | IZH3 | 2.373 | 1.047 | 0.709 |
| YLR025W | SNF7 | 2.106 | 1.099 | 1.043 |
| YLR080W | EMP46 | 3.442 | 1.22 | 1.077 |
| YLR090W | XDJ1 | 2.929 | 1.006 | 0.98 |
| YLR120C | YPS1 | 3.186 | 0.734 | 0.823 |
| YLR128W | DCN1 | 2.364 | 0.521 | 1.072 |
| YLR161W | | 2.434 | 0.762 | 1.312 |
| YLR162W | | 2.705 | 0.587 | 1.234 |
| YLR164W | | 2.442 | 1.43 | 0.974 |
| YLR177W | | 2.933 | 1.245 | 1.252 |
| YLR178C | TFS1 | 4.963 | 2.471 | 2.107 |
| YLR195C | NMT1 | 2.658 | 0.953 | 0.703 |
| YLR225C | | 6.591 | 2.378 | 1.268 |
| YLR226W | BUR2 | 3.467 | 1.198 | 0.759 |
| YLR251W | SYM1 | 4.095 | 2.015 | 1.318 |
| YLR252W | | 3.979 | 2.101 | 1.598 |
| YLR327C | TMA10 | 11.178 | 3.356 | 2.24 |
| YLR330W | CHS5 | 2.594 | 1.216 | 1.134 |
| YLR347C | KAP95 | 2.264 | 0.407 | 0.749 |
| YLR375W | STP3 | 2.124 | 1.25 | 0.865 |
| YLR392C | | 2.401 | 1.541 | 1.401 |
| YLR411W | CTR3 | 2.474 | 0.586 | 0.662 |
| YML042W | CAT2 | 2.755 | 1.375 | 1.106 |
| YML088W | UFO1 | 2.406 | 1.149 | 1.477 |
| YML100W | TSL1 | 10.747 | 1.428 | 1.32 |
| YML117W | NAB6 | 4.647 | 0.712 | 1.09 |
| YML130C | ERO1 | 5.434 | 3.369 | 3.375 |
| YML131W | | 6.445 | 3.798 | 2.613 |
| YMR033W | ARP9 | 3.379 | 2.045 | 1.464 |

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|---|---|---|---|---|
| YMR044W | IOC4 | 2.094 | 1.404 | 1.399 |
| YMR092C | AIP1 | 2.663 | 1.009 | 1.232 |
| YMR094W | CTF13 | 2.977 | 0.995 | 1.591 |
| YMR096W | SNZ1 | 11.705 | 1.547 | 1.425 |
| YMR103C | | 2.941 | 0.923 | 0.622 |
| YMR105C | PGM2 | 3.76 | 1.037 | 1.398 |
| YMR106C | YKU80 | 2.388 | 1.288 | 1.072 |
| YMR153W | NUP53 | 2.393 | 1.23 | 1.457 |
| YMR155W | | 2.466 | 0.71 | 1.098 |
| YMR160W | | 2.187 | 0.663 | 1.166 |
| YMR165C | SMP2 | 2.565 | 1.199 | 1.192 |
| YMR169C | ALD3 | 2.541 | 1.255 | 1.428 |
| YMR172C-A | | 2.758 | 1.093 | 1.403 |
| YMR172W | HOT1 | 2.288 | 1.337 | 1.368 |
| YMR181C | | 3.714 | 1.091 | 0.711 |
| YMR250W | GAD1 | 2.285 | 1.764 | 1.6 |
| YMR315W | | 3.517 | 2.05 | 1.97 |
| YMR316W | DIA1 | 2.542 | 1.779 | 1.453 |
| YNL063W | MTQ1 | 3.223 | 1.886 | 1.7 |
| YNL077W | APJ1 | 11.679 | 2.015 | 1.937 |
| YNL092W | | 2.769 | 0.953 | 1.355 |
| YNL094W | APP1 | 3.921 | 1.357 | 0.756 |
| YNL096C | RPS7B | 3.332 | 1.014 | 1.242 |
| YNL100W | | 2.184 | 1.026 | 1.326 |
| YNL104C | LEU4 | 2.689 | 0.829 | 1.003 |
| YNL115C | | 2.983 | 1.668 | 1.808 |
| YNL117W | MLS1 | 2.869 | 1.62 | 1.674 |
| YNL127W | FAR11 | 3.746 | 0.927 | 1.28 |
| YNL134C | | 11.291 | 5.736 | 6.825 |
| YNL152W | | 2.891 | 1.436 | 1.623 |
| YNL158W | PGA1 | 2.058 | 0.925 | 0.778 |
| YNL192W | CHS1 | 2.842 | 1.005 | 0.919 |
| YNL250W | RAD50 | 2.265 | 0.71 | 1.085 |
| YNL274C | GOR1 | 5.384 | 2.207 | 1.321 |
| YNL275W | BOR1 | 2.629 | 1.697 | 1.297 |
| YNL279W | PRM1 | 2.281 | 1.511 | 1.694 |
| YNL294C | RIM21 | 3.736 | 0.992 | 0.603 |
| YNL331C | AAD14 | 7.875 | 2.954 | 1.398 |
| YNL335W | DDI3 | 137.865 | 18.647 | 22.388 |
| YNR001C | CIT1 | 6.808 | 1.514 | 1.207 |
| YNR002C | FUN34 | 3.226 | 1.301 | 1.416 |
| YNR011C | PRP2 | 2.305 | 0.817 | 1.163 |
| YNR019W | ARE2 | 2.622 | 0.546 | 0.731 |
| YNR037C | RSM19 | 2.371 | 1.327 | 1.092 |
| YNR039C | ZRG17 | 2.015 | 1.31 | 1.146 |
| YNR044W | AGA1 | 2.595 | 1.462 | 1.221 |
| YNR060W | FRE4 | 17.136 | 5.196 | 2.991 |
| YNR063W | | 8.657 | 2.215 | 3.903 |
| YNR064C | | 3.66 | 1.406 | 2.155 |
| YNR065C | | 2.558 | 0.556 | 1.149 |
| YNR068C | | 16.132 | 1.469 | 3.346 |
| YOL017W | ESC8 | 3.095 | 2.101 | 1.876 |
| YOL028C | YAP7 | 3.002 | 1.082 | 1.331 |
| YOL036W | | 2.356 | 1.368 | 1.219 |
| YOL047C | | 3.967 | 1.301 | 1.242 |
| YOL058W | ARG1 | 36.656 | 2.202 | 2.421 |
| YOL059W | GPD2 | 3.551 | 0.645 | 0.976 |
| YOL060C | AMI3 | 4.744 | 0.482 | 1.107 |

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|---|---|---|---|---|
| YOL113W | SKM1 | 2.038 | 0.724 | 0.731 |
| YOL119C | MCH4 | 3.868 | 0.881 | 1.504 |
| YOL128C | | 2.233 | 0.638 | 0.665 |
| YOL165C | AAD15 | 7.905 | 3.181 | 2.535 |
| YOR003W | YSP3 | 2.083 | 1.428 | 1.246 |
| YOR014W | RTS1 | 3.49 | 1.196 | 0.566 |
| YOR019W | | 2.389 | 1.876 | 1.595 |
| YOR024W | IRC12 | 2.074 | 0.68 | 0.974 |
| YOR025W | HST3 | 2.434 | 1.059 | 0.949 |
| YOR027W | STI1 | 3.586 | 2.322 | 1.678 |
| YOR077W | RTS2 | 3.217 | 1.409 | 0.976 |
| YOR081C | TGL5 | 2.135 | 1.211 | 0.786 |
| YOR130C | ORT1 | 3.318 | 1.361 | 1.347 |
| YOR138C | RUP1 | 2.545 | 1.162 | 1.582 |
| YOR141C | ARP8 | 2.238 | 0.954 | 1.453 |
| YOR154W | SLP1 | 2.952 | 0.768 | 1.08 |
| YOR221C | MCT1 | 2.86 | 0.949 | 0.744 |
| YOR262W | | 2.102 | 1.359 | 1.125 |
| YOR267C | HRK1 | 3.515 | 0.946 | 1.382 |
| YOR273C | TPO4 | 2.411 | 0.629 | 1.164 |
| YOR286W | FMP31 | 4.131 | 1.894 | 1.406 |
| YOR299W | BUD7 | 2.192 | 1.174 | 1.028 |
| YOR328W | PDR10 | 2.005 | 0.793 | 1.116 |
| YOR329C | SCD5 | 4.498 | 1.938 | 1.478 |
| YOR363C | PIP2 | 2.062 | 1.123 | 1.293 |
| YOR371C | KRH2 | 2.298 | 1.487 | 1.531 |
| YOR386W | PHR1 | 3.122 | 1.566 | 1.531 |
| YPL005W | AEP3 | 2.105 | 1.178 | 0.859 |
| YPL019C | VTC3 | 2.148 | 0.54 | 0.949 |
| YPL046C | ELC1 | 2.249 | 0.403 | 0.673 |
| YPL055C | LGE1 | 4.105 | 1.803 | 1.805 |
| YPL088W | | 4.138 | 0.587 | 0.765 |
| YPL119C | DBP1 | 4.191 | 1.91 | 1.411 |
| YPL123C | RNY1 | 4.445 | 2.608 | 1.726 |
| YPL204W | HRR25 | 2.827 | 1.979 | 1.751 |
| YPL207W | TYW1 | 4.196 | 0.806 | 1.761 |
| YPL211W | NIP7 | 2.147 | 1.033 | 1.167 |
| YPL250C | ICY2 | 9.8 | 1.692 | 2.407 |
| YPL257W | | 2.007 | 0.527 | 1.045 |
| YPL265W | DIP5 | 3.128 | 1.008 | 1.014 |
| YPL271W | ATP15 | 4.146 | 1.006 | 1.251 |
| YPL277C | | 2.204 | 0.951 | 0.865 |
| YPR019W | CDC54 | 2.113 | 1.255 | 1.408 |
| YPR030W | CSR2 | 2.388 | 1.319 | 1.003 |
| YPR031W | NTO1 | 6.324 | 1.611 | 1.423 |
| YPR156C | TPO3 | 3.221 | 1.047 | 0.904 |
| YPR193C | HPA2 | 2.06 | 1.506 | 1.391 |
| YPR194C | OPT2 | 2.264 | 1.091 | 1.601 |